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Investigating the causal relationship between iron status and malaria in African children

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ABSTRACT

Iron deficiency (ID) and malaria are co-existing and common causes of ill health in African children. In this thesis, I aimed to determine: 1) a more accurate estimate of the burden of ID in African children by adjusting for the influence of infections on iron biomarkers; 2) whether malaria causes ID; 3) whether iron status influences malaria risk; and 4) whether a mutation in the ferroportin coding gene, *FPN* Q248H, influences iron status, anaemia and risk of malaria.

Among 4,853 children from community-based cohorts across Africa, the overall prevalence of ID was 52% using an inflammation and malaria regression-corrected estimate of ID, compared to a WHO-defined estimate of 34%. This WHO unidentified burden of ID increased with age and was highest in countries with high prevalence of inflammation and malaria, where up to a quarter of iron-deficient children were misclassified as iron replete. Transferrin saturation (TSAT) was least affected by inflammation and malaria and TSAT <11% most closely predicted the regression-corrected ID.

In Mendelian randomisation analyses, sickle cell trait, a polymorphism conferring protection against malaria, was associated with a 30% reduction in ID in 7453 malaria-exposed African children but not in 3207 non-malaria exposed African-Americans. Genetically determined malaria risk was associated with a 2.5-fold increase in ID per unit increase in log-incidence rate of malaria. This suggests that an intervention that halves the risk of malaria episodes would reduce ID by 47% in African children.

In 2683 Kenyan and Ugandan children, ID defined using either low ferritin or TSAT was associated with 33% and 21% reduction in malaria risk respectively. The *FPN* Q248H mutation which increases intracellular iron export, was associated with 23% and 25% protection from

ID and anaemia, respectively, but there was little evidence of protection from malaria. This work demonstrates the complex relationship between iron and malaria.

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TABLE OF CONTENTS

| | |
|---|-----------|
| CHAPTER ONE: LITERATURE REVIEW | 1 |
| 1.1 IRON DEFICIENCY | 2 |
| 1.1.1 Iron homeostasis | 4 |
| 1.1.2 Indicators of iron deficiency..... | 9 |
| 1.1.3 Control and management of iron deficiency..... | 13 |
| 1.2 MALARIA | 15 |
| 1.2.1 Malaria transmission | 15 |
| 1.2.2 Clinical symptoms of malaria | 16 |
| 1.2.3 Malaria diagnoses, treatment and control..... | 17 |
| 1.2.4 Genetics of malaria | 18 |
| 1.3 IRON AND MALARIA..... | 21 |
| 1.3.1 Estimating the prevalence of iron deficiency in malaria-endemic areas | 21 |
| 1.3.2 Malaria as a possible cause of iron deficiency..... | 23 |
| 1.3.3 Iron status and malaria risk..... | 29 |
| 1.3.4 The ferroportin gene, iron deficiency and risk of malaria..... | 33 |
| 1.4 MENDELIAN RANDOMISATION | 35 |
| 1.5 RESEARCH QUESTIONS RAISED DURING THIS LITERATURE REVIEW | 38 |
| 1.6 AIM AND OBJECTIVES OF THE STUDY | 39 |
| CHAPTER TWO: GENERAL METHODS | 40 |
| 2.1 STUDY POPULATIONS | 40 |
| 2.2 ASSAYS FOR IRON AND INFLAMMATORY MARKERS | 46 |
| 2.3 GENOTYPING IN THE <i>IRON_VACCGENE</i> STUDIES | 48 |
| 2.4 DEFINITIONS..... | 49 |
| 2.5 ETHICAL APPROVALS | 50 |
| CHAPTER THREE: EPIDEMIOLOGY OF IRON DEFICIENCY IN AFRICAN CHILDREN | 51 |
| 3.1 ABSTRACT | 51 |
| 3.2 INTRODUCTION..... | 52 |
| 3.3 METHODS | 54 |
| 3.3.1 Study population | 54 |
| 3.3.2 Statistical analysis | 54 |
| 3.4 RESULTS | 56 |
| 3.4.1 Characteristics of study participants | 56 |
| 3.4.2 Distribution of iron status and anaemia..... | 58 |
| 3.4.3 Age and sex differences in iron status..... | 60 |
| 3.4.4 Associations between malnutrition and iron status..... | 61 |
| 3.4.5 Associations between inflammation and malaria and iron status | 61 |
| 3.4.6 Estimating the regression-corrected prevalence of iron deficiency..... | 64 |
| 3.4.7 Misclassification of iron-deficient children increases with age, inflammation and malaria | 68 |
| 3.4.8 Diagnostic utility of iron biomarkers in predicting regression-corrected iron deficiency | 72 |
| 3.5 DISCUSSION | 74 |
| 3.6 CONCLUSION | 79 |
| CHAPTER FOUR: INVESTIGATING WHETHER MALARIA CAUSES IRON DEFICIENCY IN AFRICAN CHILDREN..... | 80 |
| 4.1 ABSTRACT | 80 |
| 4.2 INTRODUCTION..... | 81 |
| 4.3 METHODS | 82 |
| 4.3.1 Study populations..... | 82 |
| 4.3.2. Effect of sickle cell trait on malaria | 86 |
| 4.3.3. Statistical analysis | 86 |
| 4.4 RESULTS | 88 |
| 4.4.1 Characteristics of study populations..... | 88 |

| | |
|---|------------|
| 4.4.2 <i>Sickle cell trait is associated with protection against iron deficiency</i> | 90 |
| 4.4.3 <i>Malaria may cause iron deficiency through a hepcidin-mediated blockade of iron absorption and recycling</i> | 96 |
| 4.5 DISCUSSION | 98 |
| 4.6 CONCLUSION | 102 |
| CHAPTER FIVE: IRON STATUS AND ASSOCIATED MALARIA RISK AMONG AFRICAN CHILDREN | 104 |
| 5.1 ABSTRACT | 104 |
| 5.2 INTRODUCTION..... | 105 |
| 5.3 METHODS | 106 |
| 5.3.1 <i>Study population</i> | 106 |
| 5.3.2 <i>Statistical analysis</i> | 107 |
| 5.4 RESULTS | 108 |
| 5.4.1 <i>Baseline characteristics of study population</i> | 108 |
| 5.4.2 <i>Higher ferritin concentrations and TSAT are positively associated with malaria infection</i> | 109 |
| 5.4.3 <i>Iron deficiency defined using ferritin or TSAT is associated with protection against malaria risk</i> | 111 |
| 5.5 DISCUSSION | 119 |
| 5.6 CONCLUSION | 123 |
| CHAPTER SIX: FERROPORTIN Q248H IS ASSOCIATED WITH PROTECTION AGAINST IRON DEFICIENCY AND ANAEMIA BUT NOT MALARIA | 125 |
| 6.1 ABSTRACT | 125 |
| 6.2 INTRODUCTION..... | 126 |
| 6.3. METHODS | 127 |
| 6.3.1 <i>Study populations</i> | 127 |
| 6.3.2 <i>Statistical analysis</i> | 128 |
| 6.4 RESULTS | 129 |
| 6.4.1 <i>Characteristics of study participants</i> | 129 |
| 6.4.2 <i>FPN Q248H is associated with modest protection against anaemia</i> | 131 |
| 6.4.3 <i>FPN Q248H is associated with protection against iron deficiency</i> | 137 |
| 6.4.4 <i>Little evidence that FPN Q248H is associated with protection from malaria</i> | 141 |
| 6.5 DISCUSSION | 143 |
| 6.6 CONCLUSION | 145 |
| CHAPTER SEVEN: CONCLUDING REMARKS AND RECOMMENDATIONS..... | 146 |
| 7.1 LIMITATIONS AND FUTURE DIRECTIONS..... | 148 |
| REFERENCES..... | 151 |

LIST OF TABLES

| | |
|---|-----|
| Table 1.1. Definition of iron deficiency using serum ferritin | 10 |
| Table 2.1. Data from individual study sites through collaboration..... | 43 |
| Table 2.2. Laboratory assays for iron and inflammatory biomarkers by site | 47 |
| Table 3.1. Characteristics of study participants by cohort..... | 57 |
| Table 3.2. Distribution of iron status and anaemia by study cohort | 59 |
| Table 3.3. Regression-corrected and uncorrected prevalence of iron deficiency defined by additional iron biomarkers by study site | 67 |
| Table 4.1. Characteristics of study participants by site | 88 |
| Table 4.2. Effect of sickle cell trait (HbAS) on iron deficiency before and after adjusting for α -thalassaemia. | 91 |
| Table 4.3. A summary of published studies on the incidence rate ratio of sickle cell trait on uncomplicated malaria | 94 |
| Table 4.4. Log estimates used in Mendelian randomisation analyses | 94 |
| Table 5.1. Baseline characteristics of study participants | 109 |
| Table 5.2. Incidence of malaria by iron status and anaemia..... | 112 |
| Table 5.3. Cox proportional hazards regression models for predicting malaria risk..... | 114 |
| Table 5.4. A summary of observational studies examining the relationship between iron deficiency and malaria risk | 118 |
| Table 6.1. Characteristics of participants by study cohort and <i>FPN</i> Q248H mutation | 130 |
| Table 6.2. Association between <i>FPN</i> Q248H mutation and anaemia, haemolysis, and iron deficiency in community-based children | 133 |
| Table 6.3. Summary of studies examining the relationship between the <i>FPN</i> Q248H mutation and haemoglobin, ferritin, and C-reactive protein | 135 |
| Table 6.4. Association between <i>FPN</i> Q248H and uncomplicated or severe malaria..... | 142 |

LIST OF FIGURES

| | |
|---|-----|
| Figure 1.1. Relationship between malaria and iron deficiency..... | 1 |
| Figure 1.2. Systemic iron homeostasis. | 5 |
| Figure 1.3. Inhibition of hepcidin during iron deficiency and expanded erythropoiesis..... | 8 |
| Figure 1.4. The malaria - iron deficiency hypothesis. | 26 |
| Figure 1.5. Relation between iron and <i>P. falciparum</i> stages in human. | 30 |
| Figure 1.6. Comparison of a randomised controlled trial and Mendelian randomisation. | 36 |
| Figure 1.7. Conceptual Mendelian randomisation causal inference framework. | 37 |
| | |
| Figure 2.1. African study sites. | 41 |
| | |
| Figure 3.1. Geometric means for different iron biomarkers by age in years and sex..... | 60 |
| Figure 3.2. Predictors of iron biomarkers in regression models adjusted for age, sex and study site. | 62 |
| Figure 3.3. Predictors of iron biomarkers in multivariable regression models..... | 63 |
| Figure 3.4. Prevalence of estimated iron deficiency across the study sites. | 65 |
| Figure 3.5. Comparison of prevalence of iron deficiency after additional adjustments for age, sex and nutritional status..... | 66 |
| Figure 3.6. The hidden burden of iron deficiency by sex and age..... | 68 |
| Figure 3.7. Relationship between the estimated prevalence of iron deficiency and inflammation / malaria. | 70 |
| Figure 3.8. Relationship between the estimated prevalence of iron deficiency and inflammation / malaria..... | 71 |
| Figure 3.9. Receiver operating characteristic curves of the utility of iron markers in estimating adjusted prevalence of iron deficiency..... | 73 |
| Figure 3.10. Meta-analysis of optimal cutoffs of transferrin saturation in predicting regression adjusted iron deficiency. | 74 |
| | |
| Figure 4.1. Sick cell trait (HbAS) protects from iron deficiency (ID)..... | 89 |
| Figure 4.2. A meta-analysis of the effect of sickle cell trait on iron deficiency anaemia (IDA). | 91 |
| Figure 4.3. Malaria may cause iron deficiency through hepcidin-mediated blockade of iron absorption and recycling. | 92 |
| Figure 4.4. A meta-analysis of the effect of sickle cell trait on uncomplicated malaria. | 93 |
| Figure 4.5. A meta-analysis of the effects of glucose-6-phosphate-dehydrogenase deficiency (G6PD) heterozygosity on iron deficiency. | 95 |
| Figure 4.6. A meta-analysis of the effects of alpha-thalassaemia heterozygosity on iron deficiency..... | 96 |
| Figure 4.7. Geometric mean hepcidin concentrations by anaemia status..... | 97 |
| Figure 4.8. How sickle cell trait, a proxy for reduced malaria exposure, may be protecting from iron deficiency..... | 100 |
| | |
| Figure 5.1. Study design. | 107 |

| | |
|---|-----|
| Figure 5.2. Scatter plots of iron biomarkers stratified by no subsequent malaria episodes or one or more malaria episodes. | 110 |
| Figure 5.3. Adjusted incidence rate ratios for the effect of iron biomarkers on subsequent malaria episodes. | 111 |
| Figure 5.4. Kaplan-Meier curves of time to first malaria episode. | 113 |
| Figure 5.5. Effect of iron status on malaria risk over a one-year period. | 114 |
| Figure 5.6. Effect of iron status on malaria risk over a six-month period excluding children with inflammation and parasitaemia at baseline. | 115 |
| Figure 5.7. Effect of iron deficiency on malaria risk by age group. | 116 |
| Figure 5.8. Effect of iron status on malaria risk including adjustment for sickle cell trait.... | 117 |
| Figure 5.9. Meta-analysis of observational studies examining the relationship between iron deficiency and malaria risk. | 119 |

| | |
|---|-----|
| Figure 6.1. Association between <i>FPN</i> Q248H mutation and anaemia, red cell haemolysis and iron deficiency (ID)..... | 132 |
| Figure 6.2. Meta-analysis of associations of the <i>FPN</i> Q248H mutation with anaemia and haemoglobin across the study populations. | 134 |
| Figure 6.3. A meta-analysis of studies investigating the relationship between the <i>FPN</i> Q248H mutation and haemoglobin levels. | 136 |
| Figure 6.4. Meta-analysis of associations of the <i>FPN</i> Q248H mutation with iron status across the study populations. | 138 |
| Figure 6.5. Effect of the <i>FPN</i> Q248H mutation on anaemia and iron deficiency stratified by inflammation and hepcidin concentrations. | 140 |
| Figure 6.6. Effect of <i>FPN</i> Q248H variant on <i>P. falciparum</i> parasite density..... | 143 |

LIST OF ABBREVIATIONS

| | |
|----------------|--|
| ACT: | α 1-antichymotrypsin |
| ACTs: | artemisinin-based combination therapies |
| AGP: | α -1-acid glycoprotein |
| AUC: | area under curve |
| BIS: | body iron stores |
| BMP: | bone morphogenetic protein |
| BMPR: | bone morphogenetic protein receptor |
| BRINDA: | Biomarkers Reflecting Inflammation and Nutritional Determinants of Anaemia |
| CDC: | Centers for Disease Control |
| CI: | confidence interval |
| CP: | ceruloplasmin |
| CRP: | C-reactive protein |
| DCYTB: | duodenal cytochrome B |
| DDT: | dichlorodiphenyltrichloroethane |
| DMT1: | divalent metal ion transporter 1 |
| DNA: | deoxyribonucleic acid |
| DRC: | Democratic Republic of Congo |
| EDTA: | ethylenediaminetetraacetic acid |
| ELISA: | enzyme-linked immunoassay, |
| EMaBS: | Entebbe Mother and Baby Study |
| EPO: | erythropoietin |
| EPOR: | erythropoietin receptor |
| ERFE: | erythroferrone |
| ES: | effect size |
| FPN: | ferroportin gene |
| G6PD: | glucose-6-phosphate dehydrogenase |
| GWAS: | genome-wide association study |
| Hb: | haemoglobin |
| HBA1: | alpha-globin gene 1 |
| HBA2: | alpha-globin gene 2 |
| HbAC: | heterozygous haemoglobin C mutation |
| HbAS: | sickle-cell trait |

HBB: beta-globin haemoglobin gene
HbC: haemoglobin C
HbCC: homozygous haemoglobin C mutation
HbSS: sickle cell disease
HCP1: haem carrier protein 1
HDAC3: histone deacetylase 3
HEPH: hephaestin
HFE: haemochromatosis
HJV: hemojuvelin
HPLC: High Performance Liquid Chromatography
HRC: Haplotype Reference Consortium
HWE: Hardy-Weinberg equilibrium
IBD: identity-by-descent
ID: iron deficiency
IDA: iron deficiency anaemia
IL: interleukin
IPT: intermittent preventive treatment
IRR: incidence rate ratio
IRS: indoor residual spraying
ITNs: insecticide-treated bed nets
JAK2: Janus kinase 2
JHS: Jackson Heart Study
LD: linkage disequilibrium
LPS: lipopolysaccharide
LSECs: liver sinusoidal endothelial cells,
MAF: minor allele frequency
MALDI-TOF: Matrix-Assisted Laser Desorption-Ionization Time-of-Flight
MCHC: mean corpuscular haemoglobin concentrations
MCV: mean corpuscular volume
MMS: Malawi Micronutrient Survey
MNPs: multiple micronutrients powders
N/A: not applicable
n/a: not available

NTBI: non-transferrin bound iron

OR: odds ratio

OTR: Oxford Tropical Research

PCR: polymerase chain reaction

PfPR: P. falciparum prevalence

PI: principal investigator

Q248H: glutamine to histidine at position 248 of ferroportin gene

RBCs: red blood cells

RDT: rapid diagnostic test

ROCs: receiver operating characteristics curves

ROS: reactive oxygen species

RR: risk ratio

SMAD: sons of mothers against decapentaplegic

SMC: seasonal malaria chemoprevention

SNPs: single nucleotide polymorphisms

STAT3: signal transducer and activator of transcription 3

sTfR: soluble transferrin receptors

TF: transferrin

TFR1: transferrin receptor 1

TFR2: transferrin receptor 2

TIBC: total iron-binding capacity

TMPRSS6: transmembrane serine protease 6

TNF- α : tumor necrosis factor- α

TSAT: transferrin saturation

UIBC: unsaturated iron binding capacity

WHO: World Health Organization

YLD: years lived with disability

ZPP: zinc protoporphyrin

LIST OF PUBLICATIONS FROM THIS THESIS

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3. J. M. Muriuki, A. J. Mentzer, G. Band, J. J. Gilchrist, T. Carstensen, S. A. Lule, M. M. Goheen, F. Joof, W. Kimita, C. Cutland, A. Diarra, A. Rautanen, C. Pomilla, D. Gurdasani, K. Rockett, N. Mturi, F. M. Ndungu, J. A. G. Scott, S. B. Sirima, A. Morovat, A. M. Prentice, S. A. Madhi, E. L. Webb, A. M. Elliott, P. Bejon, M. S. Sandhu, A. V. S. Hill, D. P. Kwiatkowski, N. Thomas, C. Cerami, S. H. Atkinson, The ferroportin Q248H mutation protects from anemia, but not malaria or bacteremia. *Science Advances* **5**, eaaw0109 (2019).
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CHAPTER ONE: LITERATURE REVIEW

Both iron deficiency and malaria are important co-existing public health problems in African children. Their relationship is complex and may be bidirectional (Figure 1.1). Iron is a vital nutrient for the malaria parasite. Conversely, malaria may mediate blockade of intestinal iron absorption and macrophage iron recycling by up-regulating the iron-regulatory hormone, hepcidin. This complex relationship between iron deficiency and malaria may account for a large proportion of ill health in African children. Iron supplementation is recommended for children at risk of iron deficiency (World Health Organization, 2016). However, it is unclear whether improved body iron status causes increased risk of malaria infections. Conversely, little is known about whether malaria causes iron deficiency. In this chapter I review the literature on iron deficiency, malaria, putative causal relationships between iron deficiency and malaria, and Mendelian randomisation as an approach for understanding that complex relationship.

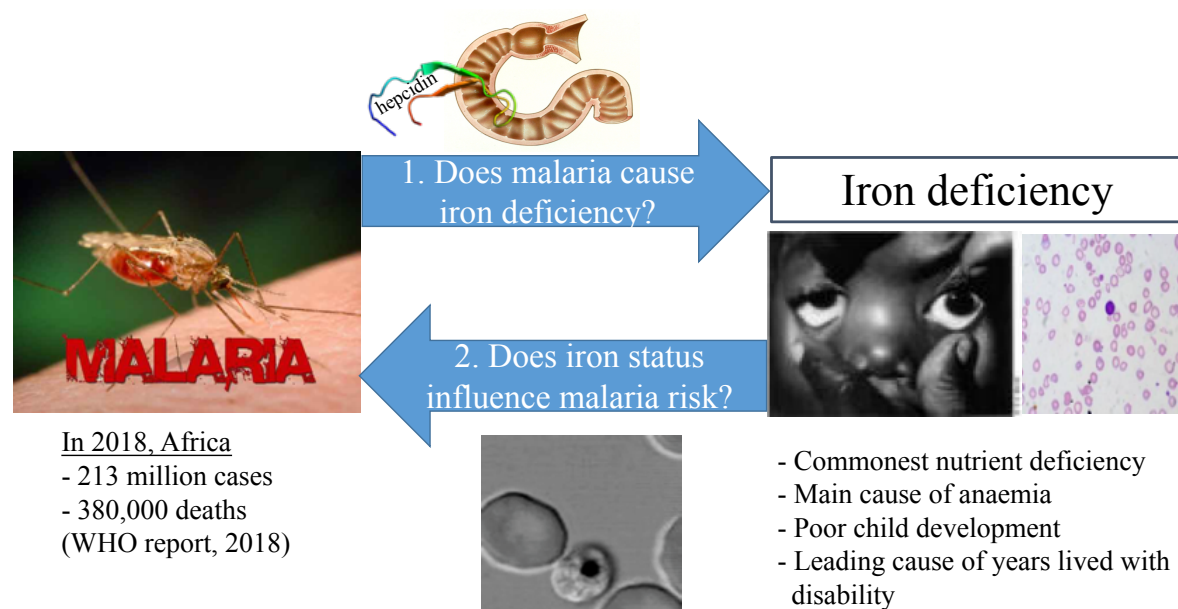


Figure 1.1. Relationship between malaria and iron deficiency.

Malaria may cause iron deficiency by up-regulating production of hepcidin by the liver blocking iron absorption. Conversely, the malaria parasite requires iron to grow and multiply, thus improving iron status may increase malaria risk.

1.1 Iron deficiency

Iron deficiency is the most prevalent nutritional deficiency worldwide. It accounts for over 60% of all anaemia cases and iron deficiency anaemia is estimated to affect over 1.2 billion people worldwide (Kassebaum and GBD 2013 Anemia Collaborators, 2016; GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017). The global prevalence of iron deficiency in the absence of anaemia is unknown but is likely to be more frequent – at least double that of iron deficiency anaemia. Iron deficiency is associated with poor brain development and long-term impairment of behavioural and cognitive performance (Beard, 2007; McCann and Ames, 2007; Lozoff, 2011; Doom and Georgieff, 2014). Importantly, iron deficiency is estimated to be a leading cause of years lived with disability (YLD) in children (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017). In Africa, iron deficiency is estimated to be the leading cause of YLD in 30 out of the 46 sub-Saharan African countries (GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017).

Iron deficiency is defined as insufficient body iron for maintaining normal physiological functions. It occurs in three stages: 1) depletion in body iron stores, 2) iron-deficient erythropoiesis or functional iron deficiency, and 3) iron deficiency anaemia (Camaschella, 2015). Depletion in iron stores is reflected by reduced serum ferritin concentrations. Iron deficient erythropoiesis is characterised by low serum iron and reduced iron incorporation into red blood cells (RBCs). Iron deficiency is often confused for iron deficiency anaemia because most of the body iron needs are for production of RBCs and reduction in RBCs (or anaemia) is a more evident sign of iron deficiency. However, iron deficiency may occur long before anaemia becomes evident and is broad encompassing depleted iron even in other tissues such as muscle and brain. Common symptoms of iron deficiency include pallor (on skin,

conjunctivae and nail beds), weakness, difficult breathing, dizziness, headache, hair loss, dry/rough skin, pica, and restless leg syndrome (Powers and Buchanan, 2014).

There are multiple causes of iron deficiency in children including low dietary iron intake, impaired iron absorption, excessive iron losses, increased iron need for growth, or genetic factors. The efficiency of intestinal iron absorption can be determined by the type of iron in foods (haem or non-haem), presence of iron absorption inhibitors or enhancers, infections, iron stores or iron requirements (these are discussed in the next Sub-section 1.1.1 on iron homeostasis). As children grow, iron requirements are increased due to rapid increase in erythroid and muscle mass (Ziegler, Nelson and Jeter, 2014). Meeting this iron need is also critical for proper development of the brain and other body organs. In developing countries chronic infections or malnutrition-associated inflammation are common and this may be an underlying cause of iron deficiency.

Maternal iron status and newborn's gestational age may also be a major determinant of a child's iron status after birth. Higher maternal iron status or supplementation (especially when the mother is iron deficient) has been associated with reduced risks of infant iron deficiency, preterm births, and birthweight (Colomer *et al.*, 1990; Scholl, 2005; Peña-Rosas *et al.*, 2015; Mwangi, Prentice and Verhoef, 2017). Importantly, a fetus acquires most of its iron during the last trimester of pregnancy (Fisher and Nemeth, 2017). Preterm babies are therefore more likely to be born with low iron stores (Moreno-fernandez *et al.*, 2019). Since high iron stores endowment at birth is necessary for rapid growth during infancy, preterm babies are more likely to develop iron deficiency (Moreno-fernandez *et al.*, 2019).

1.1.1 Iron homeostasis

Nearly all living organisms require iron for survival (Aisen, Enns and Wessling-Resnick, 2001). In humans, iron is an essential element involved in key body functions such as oxygen and electron transport, deoxyribonucleic acid (DNA) synthesis, immune function, and also growth and development (Atamna, Walter and Ames, 2002; McCann and Ames, 2007). However, in excess or in its free state, iron is toxic to body tissues because it readily accepts or donates electrons forming highly reactive oxygen species (ROS). Moreover, excess body iron may be utilised by pathogens causing disease. Since there is no iron excretory means in mammals, the human body has developed mechanisms to tightly regulate iron. This iron regulation is either at systemic or cellular level. In this review, I will focus on systemic iron regulation.

Systemic iron regulation involves mechanisms that regulate absorption of dietary iron and its distribution in the bloodstream. There are two types of absorbable dietary iron for humans: haem and non-haem iron. Haem iron (from meat, fish, and poultry) is more efficiently absorbed compared to iron sourced from plant foods (non-haem). In meat, 30-60% of iron is haem and 15-35% of this iron is absorbed (Cook and Monsen, 1976; Monsen *et al.*, 1978). In contrast, absorption of non-haem iron from plant-based foods is <10% (Monsen *et al.*, 1978). Moreover, phytates and polyphenols (tannins) that are found in grains and tea inhibit iron absorption (Monsen *et al.*, 1978). At the intestinal brush border, haem iron is absorbed through a haem transporter (possibly Haem Carrier Protein 1 (HCP1)) (Shayeghi *et al.*, 2005) while non-haem iron passes through the divalent metal ion transporter 1 (DMT1) after conversion from ferric (Fe^{3+}) form to ferrous (Fe^{2+}) iron by duodenal cytochrome B (DCYTB), a ferrireductase (Gunshin *et al.*, 1997; McKie *et al.*, 2001) (Figure 1.2).

Once inside the enterocyte, iron is either stored as ferritin (and lost once the cell is exfoliated) or is transported across the basolateral membrane into the blood through ferroportin. In blood, ferrous iron is immediately oxidised by a ferroxidase to ferric iron and is transported to body tissue bound to transferrin and presented to cells through transferrin receptor 1 (TFR1). Iron is mainly transported to the bone marrow to produce RBCs (erythropoiesis). Once RBCs age or are haemolysed, they are taken up by splenic and liver macrophages and approximately 25mg of iron per day is recycled back into the circulation. Ferroportin is the sole known protein that exports intracellular iron mainly from enterocytes, macrophages and hepatocytes (Abboud and Haile, 2000; Donovan *et al.*, 2000; McKie *et al.*, 2000). Thus, ferroportin plays a major role in systemic iron homeostasis.

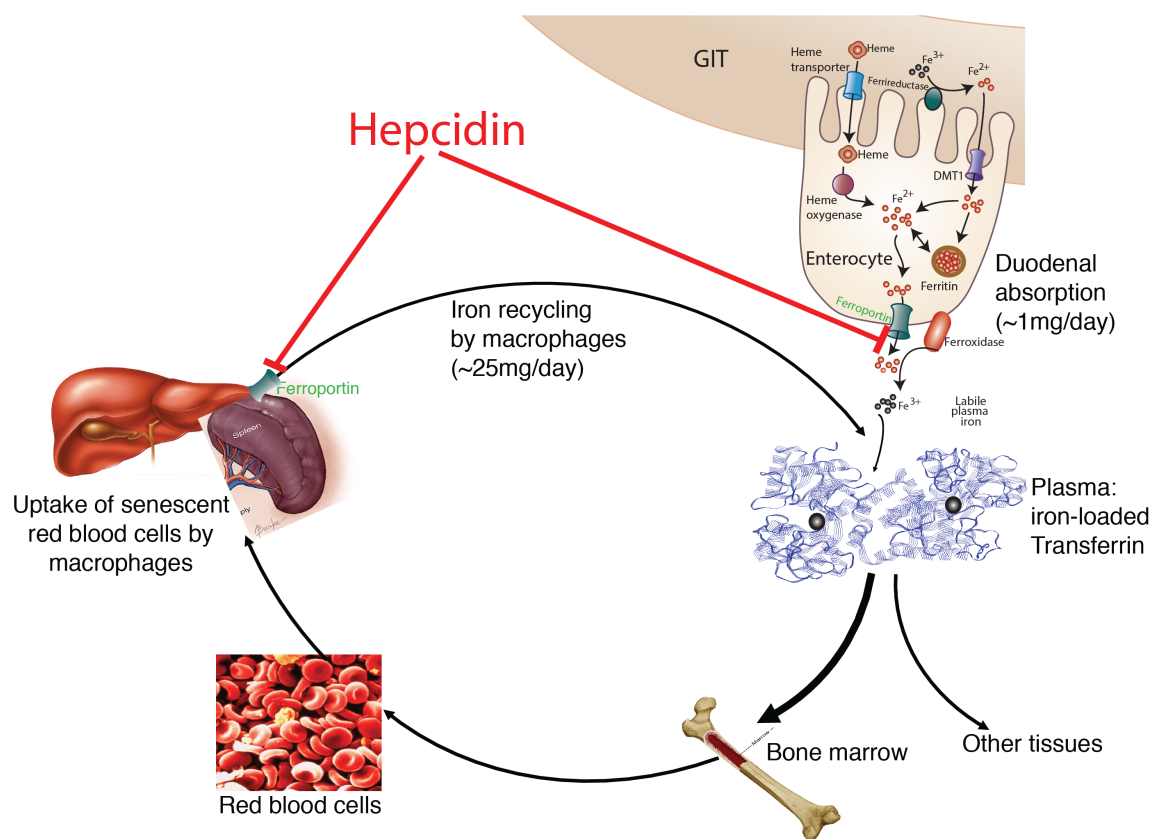


Figure 1.2. Systemic iron homeostasis.

Approximately 1mg of iron per day is absorbed through duodenal enterocytes and is exported into the circulation through ferroportin. In blood, iron is bound to transferrin and is transported to body tissues, mainly to the bone marrow to produce RBCs. When RBCs age, they are taken up by splenic or liver macrophages and approximately 25mg of iron per day is recycled back into the circulation. During iron overload or infection or inflammation,

production of hepcidin by the liver is up-regulated. Hepcidin degrades ferroportin on enterocytes and macrophages blocking absorption and recycling of iron. Conversely, during iron deficiency and expanded erythropoiesis, hepcidin production is suppressed to increase absorption and recycling of iron. Modified from (Spottiswoode and Fried, 2012).

Systemic iron homeostasis is regulated by hepcidin, a hormone that is produced mainly by the liver. Since excess of iron is toxic to humans and is a nutrient to pathogens, production of hepcidin is up-regulated in iron overload or an inflammatory process or infection (Ganz, 2011). Hepcidin prevents iron absorption and recycling through occlusion and degradation of ferroportin on enterocytes and macrophages (Nemeth, Tuttle, *et al.*, 2004; Aschemeyer *et al.*, 2018). Hepcidin also degrades and inhibits the transcription of DMT1, thus further blocking iron absorption through the duodenal enterocytes (Mena *et al.*, 2008; Brasselagnel *et al.*, 2011). Conversely, during iron deficiency, expanded erythropoiesis, or hypoxic conditions, hepcidin concentrations are down-regulated so that more iron can be absorbed and recycled (Ganz, 2011).

Since the discovery of hepcidin's role in iron homeostasis in 2001 (Nicolas *et al.*, 2001; Pigeon *et al.*, 2001), evidence has been accumulating on the mechanisms of iron-regulation by hepcidin. Expression of hepcidin in the liver is mainly regulated by the BMP-SMAD (bone morphogenetic protein – sons of mothers against decapentaplegic) signaling pathway. During iron deficiency, transcription of BMP2 and BMP6 in liver sinusoidal endothelial cells is down-regulated (recently reviewed in (Silvestri *et al.*, 2019)). However, the mechanism through which liver sinusoidal endothelial cells sense iron concentrations remains unclear. Regulatory SMADs 1, 5, and 8 are phosphorylated by BMPs then form a complex with SMAD 4 (the common mediator) and are translocated to the nucleus to stimulate target gene expression (Silvestri *et al.*, 2019).

Mutations in the haemojuvelin (*HJV*) gene and the haemochromatosis (*HFE*) and transferrin receptor 2 (*TFR2*) proteins are also important in the activation of the BMP-SMAD signaling pathway. During iron deficiency, the BMPs coreceptor *HJV* is cleaved from the surface of hepatocytes by transmembrane serine protease 6 (*TMPRSS6*) suppressing hepcidin production (Babitt *et al.*, 2006; Silvestri *et al.*, 2008; Lakhal *et al.*, 2011). Moreover, in the absence of diferric transferrin, *TFR2*, a circulating iron sensor, is cleaved from the surface of hepatocytes and erythroid cells down-regulating hepcidin production (Pagani *et al.*, 2015). Inactivation of *BMP6* in *TFR2* or *HFE* knock-out mice has been shown to suppress hepcidin production through unknown mechanisms (Latour *et al.*, 2016).

Similarly, hepcidin is suppressed during expanded erythropoiesis due to increased erythropoietin which stimulates production of erythroferrone to block hepcidin production (Kautz *et al.*, 2014). Interestingly, the inhibitory effect of erythroferrone is attenuated in mice lacking *SMAD 1* and *5* suggesting that erythroferrone requires *SMAD* proteins to block hepcidin production (Wang *et al.*, 2017). However, the mechanism through which erythroferrone inhibits hepcidin production through the *BMP* pathway remains uncertain. Taken together, these mechanisms serve to explain how hepcidin concentrations are reduced during iron deficiency and expanded erythropoiesis to increase iron absorption and recycling (Figure 1.3).

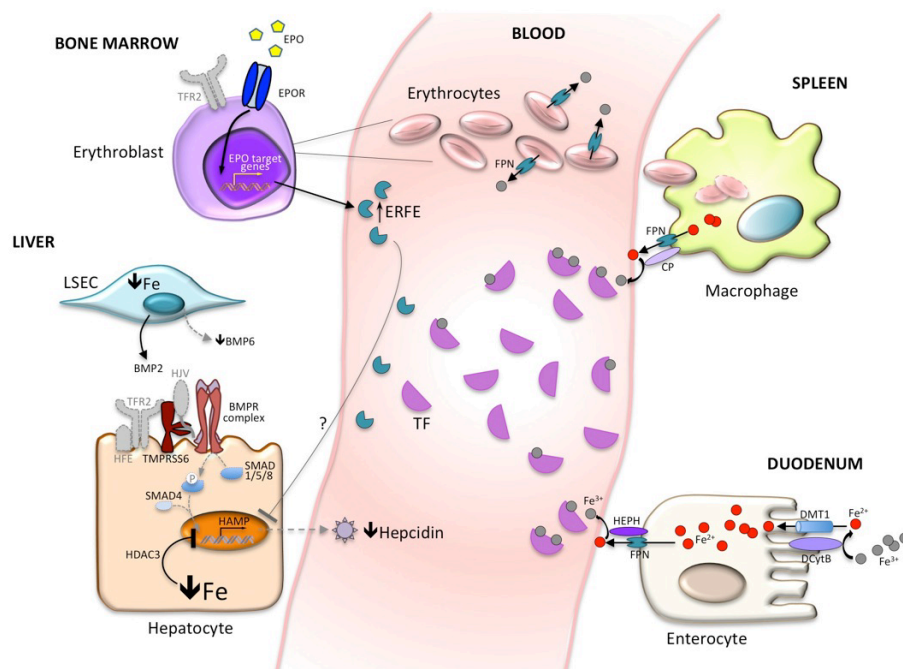


Figure 1.3. Inhibition of hepcidin during iron deficiency and expanded erythropoiesis.

*Low iron concentrations lower the BMP-SMAD signaling pathway. Production of BMPs in the liver sinusoidal endothelial cells (LSECs) is decreased. Haemojuvelin (HJV) a coreceptor for BMPs is cleaved by the transmembrane serine protease 6 (TMPRSS6). Additionally, transferrin receptor 2 (TFR2) is removed from the cell surface in the absence of diferric transferrin (TF). Furthermore, histone deacetylase 3 (HDAC3) erases markers of hepcidin activation suppressing hepcidin production (Pasricha *et al.*, 2017). Reduced hepcidin concentrations increase iron absorption by duodenal enterocytes and recycling by macrophages. In expanded erythropoiesis, erythropoietin (EPO) is increased and stimulates production of erythroferrone (ERFE) which blocks hepcidin production. BMPR, BMP receptor; CP, ceruloplasmin; DCYTB, duodenal cytochrome B; DMT1, divalent metal transporter 1; EPOR, EPO receptor; HEPH, hephaestin. Adapted from (Camaschella, 2019).*

During inflammation, hepcidin is up-regulated through a JAK2-STAT3 (Janus kinase 2 - signal transducer and activator of transcription 3) signaling pathway. Lipopolysaccharide (LPS), which is abundant on the outer membrane of Gram-negative bacteria induces production of inflammatory cytokines including interleukins, IL-6, IL-1 α , and IL-1 β , that up-regulate hepcidin production (Lee *et al.*, 2005). LPS binds on Toll-like receptor 4 in macrophages mainly stimulating production of IL-6. IL-6 binds to its receptor, glycoprotein 130 in hepatocytes to activate the JAK2-STAT3 signaling pathway. Once STAT3 is phosphorylated, it translocates into the nucleus where it binds the STAT3 responsive element on the hepcidin

promoter activating expression of hepcidin (Verga-Falzacappa *et al.*, 2007). LPS may also induce hepcidin expression through the BMP-SMAD pathway by stabilising SMAD4 (Layoun *et al.*, 2018).

1.1.2 Indicators of iron deficiency

To combat iron deficiency, it is important to accurately measure iron status. The gold standard method for estimating body iron status is to stain bone marrow aspirate for iron (Phiri, Calis, Kachala, *et al.*, 2009). However, this method is invasive and expensive and therefore impractical to perform during population surveys. A number of iron markers are used to indicate iron status but they all have limitations in estimating iron deficiency especially in areas of high infectious burden including sub-Saharan Africa (Aguilar *et al.*, 2012). Conventional indicators of iron status include serum ferritin, soluble transferrin receptors (sTfR), transferrin saturation (TSAT), ferritin index, body iron stores, and zinc protoporphyrin (ZPP). Although haemoglobin defines anaemia, its usefulness in defining iron deficiency is limited due to its low sensitivity and specificity in determining iron status since it is influenced by multiple aetiological factors (Cook and Smith, 1976; Foote *et al.*, 2013). Next, I will discuss each of the conventional iron biomarkers.

1.1.2.1 Serum ferritin

Ferritin is a hollow globular intracellular protein that stores body iron. Small amounts of ferritin are normally secreted into the serum. A measure of serum ferritin sensitively indicates body iron stores in the absence of an infection or inflammation. However, in the presence of infection, the concentration of ferritin may rise even when there are low iron stores since it is an acute phase reactant protein (Feelders *et al.*, 1998). Therefore, ferritin may only be a reliable indicator of iron deficiency in the absence of infection (Ali, Luxton and Walker, 1978). Since

the detection of ferritin in serum in 1972 (Addison *et al.*, 1972) and development of a sensitive immunoassay for ferritin in 1975 (Jacobs and Worwood, 1975), serum ferritin has been used to identify iron deficient individuals.

Defining iron deficiency using serum ferritin in areas of high infectious disease burden is challenging. Based on clinical studies and in the absence of infection or inflammation, the World Health Organization (WHO) defines iron deficiency as serum ferritin $< 12 \mu\text{g/L}$ in children < 5 years or $< 15 \mu\text{g/L}$ in children ≥ 5 years (Table 1.1) (World Health Organization, 2011b). In the presence of inflammation (defined as C-reactive protein (CRP) $> 5 \text{ mg/L}$ or α_1 -antichymotrypsin (ACT) $> 0.6 \text{ g/L}$ or α -1-acid glycoprotein (AGP) $> 1 \text{ g/L}$), the WHO defines iron deficiency as serum ferritin $< 30 \mu\text{g/L}$ but only in children < 5 years (World Health Organization, 2011b). However, there is little consensus about this inflammation-adjustment. Recent evidence shows that an inflammatory process may influence serum ferritin levels long before an individual is considered clinically inflamed (at the above cut-offs) (Namaste, Rohner, *et al.*, 2017). Another proposal for estimating the burden of iron deficiency in sites with high incidence of infection is to exclude individuals with inflammation (World Health Organization and Centers for Disease Control, 2007). However, this approach would reduce sample size, may not reflect population prevalence of iron deficiency, and uses an arbitrary cut-off to define inflammation. Thus, the current WHO definition of iron deficiency is likely to underestimate the ‘true’ burden of iron deficiency in sub-Saharan Africa.

Table 1.1. Definition of iron deficiency using serum ferritin

| | Serum ferritin ($\mu\text{g/L}$) | | | |
|---|------------------------------------|--------|-------------------------|---------|
| | Less than 5 years of age | | 5 years of age or older | |
| | Male | Female | Male | Female |
| Depleted iron stores | < 12 | < 12 | < 15 | < 15 |
| Depleted iron stores in the presence of infection | < 30 | < 30 | - | - |
| Severe risk of iron overload (adults) | - | - | > 200 | > 150 |

Table adapted from (World Health Organization, 2011b)

1.1.2.2 Soluble transferrin receptors

Iron is normally presented to cells through transferrin receptors on the cell surface. Over 80% of transferrin receptors are found on erythroid cells (Huebers and Finch, 1987). The transferrin receptor binds transferrin-bound iron and is endocytosed to release iron into cell cytosol before being recycled back on the cell surface (Klausner *et al.*, 1983). In 1987, sTfR was measured in human serum and the number of transferrin receptors were shown to reflect iron requirements for erythropoiesis (Kohgo *et al.*, 1986, 1987). sTfR is a truncated form of tissue transferrin receptor (Shih *et al.*, 1990). sTfR was proposed as an additional marker of iron deficiency especially in areas where inflammation is prevalent since it is only mildly increased during inflammation (Ferguson *et al.*, 1992; Pettersson, Kivivuori and Siimes, 1994; Punnonen, Irjala and Rajamäki, 1994; Mast *et al.*, 1998). However, the utility of sTfR as a measure of iron status may be complicated in African populations since it may be increased by malaria, even in asymptomatic infection, and by haemolytic conditions such as sickle cell disease, thalassaemia, and glucose-6-phosphate dehydrogenase (G6PD) deficiency (Menendez *et al.*, 2001; Verhoef *et al.*, 2001; Rohner *et al.*, 2017). Moreover, there are no standardised sTfR reference assays or cut-offs to define iron deficiency. Computations of total body iron stores (BIS, calculated as $-\log_{10}((\text{sTfR in mg/L} \times 1000) / \text{ferritin in } \mu\text{g/L}) - 2.8229) / 0.1207$ (Cook, Flowers and Skikne, 2003)) and ferritin index (calculated as $\text{sTfR in mg/L} / \log_{10}(\text{ferritin in } \mu\text{g/L})$ (Phiri, Calis, Siyasiya, *et al.*, 2009)) have previously been proposed and suggested to improve the diagnostic utility of these two iron markers. However, since both ferritin and sTfR have the above discussed limitations, the utility of BIS and ferritin index in correctly defining iron deficiency in African populations may also be limited.

1.1.2.3 Transferrin saturation

Iron is ferried in circulation mainly bound to transferrin, a protein that is mainly synthesised by the liver. TSAT is the ratio of serum iron to total iron-binding capacity (TIBC), which is the sum of all iron binding sites of transferrin (Yamanishi *et al.*, 2003). Each mole of transferrin binds two moles of iron and it is estimated that a transferrin concentration of 1g/L would carry 25.1 $\mu\text{mol/L}$ of iron. Thus, TSAT (expressed as a percentage) can be calculated as (serum iron in $\mu\text{mol/L}$ divided by transferrin in g/L) $\times 25.1 \times 100$) (Yamanishi *et al.*, 2003). That is, the percentage of iron binding sites that are occupied by iron. Under normal circumstances only a third of transferrin sites bind iron.

Since TSAT reflects the amount of circulating iron, it is thought to be a better marker of iron status compared to serum iron alone. However, TSAT is not without limitations since transferrin synthesis may be decreased during malnutrition (Agarwal *et al.*, 1981), and serum iron is prone to diurnal fluctuations from dietary iron intake or recycled iron (World Health Organization and Centers for Disease Control, 2007). Nevertheless, TSAT is normally used as a marker of iron deficiency especially in adults where TSAT in the range of 20 - 50% is considered normal (World Health Organization and Centers for Disease Control, 2007). However, little is known about normal TSAT values in young children although values are likely to be lower compared to TSAT values in adults (Milman and Cohn, 1984; Higgins, Chan and Adeli, 2017).

1.1.2.4 Zinc protoporphyrin

During the last stage of haemoglobin formation, iron is incorporated into protoporphyrin (the immediate precursor of haem) by ferrochetalase. A lack of iron leads to incorporation of zinc in place of iron forming ZPP. Thus, increased concentration of ZPP is an indication of severe

iron deficiency, particularly iron-deficient erythropoiesis (Labbé and Dewanji, 2004). A ZPP $>80 \mu\text{mol/mol}$ haem is considered to indicate iron deficiency (World Health Organization and Centers for Disease Control, 2007). However, high concentrations of ZPP may indicate lead poisoning rather than iron deficiency (Labbé and Dewanji, 2004). Moreover, ZPP may also be raised by the acute phase response to infections or inflammation or other causes that limit iron supply to erythroblasts thus limiting its utility in populations with a high burden of infections (Stoltzfus *et al.*, 1997, 2000).

1.1.3 Control and management of iron deficiency

The long-term adverse effects of iron deficiency including impaired brain development (McCann and Ames, 2007) call for urgent need to treat and control iron deficiency. However, despite the many efforts to combat iron deficiency, there has been little success especially in areas with high infectious disease burden such as many parts of sub-Saharan Africa. This failure to control iron deficiency may partly be attributable to the fact that it is challenging to accurately estimate the burden of iron deficiency in populations where infections are prevalent (Aguilar *et al.*, 2012). Moreover, low haemoglobin concentrations are normally used to monitor the need for and progress of nutritional iron interventions although iron deficiency without anaemia may be more common (World Health Organization, 2001) and there are multiple causes of anaemia (Foote *et al.*, 2013).

A number of approaches are applied to directly or indirectly control iron deficiency. At present, the WHO recommends daily oral iron supplementation (10 -12.5 mg of elemental iron) in children living in areas where the prevalence of anaemia is $\geq 40\%$ (World Health Organization, 2016). In malaria-endemic areas, the recommendation is that iron supplementation should be provided in conjunction with malaria control and treatment measures (World Health

Organization, 2016). This recommendation followed a Cochrane review that showed that iron supplements do not worsen malaria infection when malaria control and treatment measures are in place (Neuberger *et al.*, 2016). However, questions arise on coverage or how effective malaria control and treatment services should be to warrant safe iron supplementation. I will discuss more of this conundrum in Section 1.3. Iron supplementation may also increase hepcidin concentrations leading to poor iron absorption (Moretti *et al.*, 2015).

Fortification of foods and use of iron-enriched multiple micronutrient powders (MNPs or “Sprinkles”) are other alternative strategies for managing and controlling iron deficiency. Fortification is performed on staple foods (such as maize flour, wheat and rice) and condiments using highly bioavailable iron compounds such as ferrous sulfate and fumarate (Allen *et al.*, 2006). In MNPs, iron is encapsulated in a lipid layer and supplied as single-dose sachets ready for use at home. In a Cochrane review home fortification with MNPs was associated with a 51% decrease in iron deficiency among children < 2 years old (De-Regil *et al.*, 2013), however, studies have shown that the iron content in MNPs or fortificants may disturb gut microbiota leading to gastrointestinal disorders (Zimmermann *et al.*, 2010; Jaeggi *et al.*, 2015; Tang *et al.*, 2017).

There are other approaches that are used to control iron deficiency in children. Iron supplementation during pregnancy is thought to improve a child’s iron stores at birth and birthweight although a Cochrane review reported little benefit (Peña-Rosas *et al.*, 2015). Periodic use of anthelmintic treatments, such as albendazole and praziquantel, was also thought to improve haemoglobin concentrations (Gulani *et al.*, 2007), but a more recent Cochrane review reported insufficient evidence (Taylor-Robinson *et al.*, 2015). In addition, delayed clamping of the umbilical cord improves iron stores at birth by allowing additional transfer of

placental iron to the newborn although this may increase risk of neonatal jaundice and polycythaemia (McDonald *et al.*, 2014). Absorption of dietary non-haem iron can also be improved by the intake of enhancers such as ascorbic acid present in many fruits and by reducing intake of inhibitors such as phytates and polyphenols (Monsen *et al.*, 1978). This review suggests that management of iron deficiency is difficult especially in populations where diets are poor and infections are prevalent.

1.2 Malaria

For millennia, malaria has remained a major public health burden particularly in sub-Saharan Africa. In 2018, the WHO estimated that there are 228 million cases of malaria (up from 219 million cases in 2017) resulting in over 405,000 deaths globally (World Health Organization, 2019). Of the total deaths, 94% occurred in sub-Saharan Africa and 67% were in children under the age of five years. Although the world has seen a substantial decline in malaria burden, especially since the year 2000, there has been no significant progress in reducing malaria cases since the year 2015 (World Health Organization, 2019).

1.2.1 Malaria transmission

The life cycle of the malaria parasite involves the mammalian host and a female *Anopheles* mosquito as a transmission vector. The infected mosquito injects sporozoites into the human host and the sporozoites are quickly transported to the liver where they invade hepatocytes, proliferate, and differentiate into merozoites that then burst out into the bloodstream and invade RBCs (Meibalan and Marti, 2017). Within RBCs, the parasite undergoes asexual multiplication (for a period of 48 hours for *Plasmodium falciparum*) from ring stage to trophozoites, which then form schizonts. The schizonts mature and burst out of the RBC as merozoites that then invade new RBCs and start the erythrocytic cycle again. Clinical symptoms of malaria,

especially fever, are associated with this burst of RBCs. Some schizonts sexually differentiate into male and female gametocytes that are then taken up by a feeding mosquito to begin the vector stage. Within the mosquito the male and female microgametes differentiate and fuse to form a zygote which then differentiates and matures into sporozoites ready for inoculation into another human host (Meibalan and Marti, 2017).

Malaria in humans is caused by five species of *Plasmodium*, *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. *P. falciparum* accounts for the majority of infections and deaths in Africa. In 2018, *P. falciparum* accounted for 99.7% of all malaria infections in sub-Saharan Africa (World Health Organization, 2019). *P. vivax* accounts for the highest number of cases of malaria outside Africa (>50%) and only <10% of cases in sub-Saharan Africa (Mendis *et al.*, 2001). *P. ovale* and *P. malariae* may be more common in sub-Saharan Africa than previously estimated since they are often undetected by light microscopy although infection is less severe compared to *P. falciparum* (Doderer-Lang *et al.*, 2014). *P. knowlesi*, whose natural host is non-human primates has emerged as infectious in humans, particularly in South East Asia, but this has not yet been reported in Africa (Moyes *et al.*, 2014). Thus, this thesis focuses on *P. falciparum* malaria infection.

1.2.2 Clinical symptoms of malaria

P. falciparum malaria infection may present as either uncomplicated febrile malaria, severe malaria, or asymptomatic malaria depending on host susceptibility, immunity, and efficacy of antimalarial drugs. Uncomplicated *P. falciparum* malaria is characterised by symptoms that include fever, chills, fatigue, headache, malaise, back and limb pains, dizziness, and vomiting. When left untreated, uncomplicated *P. falciparum* malaria may progress to severe malaria and death. Severe malaria may mainly manifest as severe anaemia, cerebral malaria / impaired

consciousness, and/or respiratory distress (Marsh *et al.*, 1995). Asymptomatic malaria presents as parasitaemia in the absence of fever or other clinical symptoms of malaria. In malaria endemic regions, such as most of sub-Saharan Africa, prolonged exposure to malaria infection results in development of partial immunity against the parasite (Bejon *et al.*, 2009). This partial immunity to malaria may explain the existence of asymptomatic parasitaemia although the mechanisms of protection remain unclear. Although asymptomatic individuals carry parasites and maintain a reservoir for malaria transmission it remains controversial about whether they should be treated or not (Chen *et al.*, 2016). Susceptibility to clinical *P. falciparum* malaria infection in individuals living in the same geographic area may be partially explained by acquired immunity or genetic predisposition. In Sub-section 1.2.4, I will focus on malaria susceptibility due to genetic variations.

1.2.3 Malaria diagnoses, treatment and control

In routine clinical practice, malaria parasitaemia can be confirmed using light microscopy or rapid diagnostic test (RDT). Treatment regimens for malaria depend on disease severity. Uncomplicated *P. falciparum* malaria is treated using a three-day regimen of artemisinin-based combination therapies (ACTs). Severe malaria is managed using intravenous or intramuscular artesunate for at least 24 hours followed by a three-day course of ACTs (Dondorp *et al.*, 2010). There are reports of *P. falciparum* developing resistance to ACTs in South East Asia but none in Africa (Ashley *et al.*, 2014). Despite the effectiveness of ACTs in treating malaria, only 20% of malaria-confirmed children less than five years of age in Africa had access to ACTs in 2015 (Bennett *et al.*, 2017).

An integrated approach has historically been applied to control malaria. Approaches such as use of insecticide-treated bed nets (ITNs), indoor residual spraying (IRS) with

dichlorodiphenyltrichloroethane (DDT), larval habitat control, intermittent preventive treatment (IPT) during pregnancy and infancy, and seasonal malaria chemoprevention (SMC) are used in malaria control. Mass drug administration of antimalarials is another malaria control approach that has been shown to substantially reduce the initial risk of malaria infection especially in areas of low malaria transmission (Poirot *et al.*, 2013; von Seidlein *et al.*, 2019). A promising endeavor towards malaria control is the development of malaria vaccines. At present, only RTS,S has been approved for vaccination with an efficacy of approximately 30% in children (The RTSS Clinical Trials Partnership, 2012) and a waning efficacy over time (The RTSS Clinical Trials Partnership, 2014). Recently, ivermectin, an anti-parasitic drug, was reported to reduce residual malaria transmission and thus may have potential for use in integrated management and control of malaria (Foy *et al.*, 2019).

1.2.4 Genetics of malaria

A number of genetic polymorphisms in the human genome have been evolutionarily selected due to malaria exposure. These can be classified into haemoglobinopathies, enzymatic deficiencies, blood groups, and RBC membrane defects among others. In this chapter, I will focus the review on the common polymorphisms that confer resistance to malaria in sub-Saharan Africa. One common example of evolutionary selective pressure is sickle-cell trait (HbAS), the major haemoglobinopathy. HbAS results from inheriting one abnormal allele (substitution of glycine with valine at amino acid position 6) of beta-globin (*HBB*) gene (i.e. presence of one abnormal beta-chain in the haemoglobin). Inheritance of two abnormal alleles of *HBB* leads to sickle cell disease (HbSS). The prevalence of HbSS is normally low because of high early mortality in children born with this condition (Rees, Williams and Gladwin, 2010). HbSS is associated with sickling of RBCs, which leads to adverse effects such as microvascular obstruction and haemolysis.

Unlike HbSS, HbAS is normally asymptomatic and has been associated with partial protection against both uncomplicated and severe malaria. For example, in a meta-analysis of longitudinal studies, HbAS was associated with 31% protection from uncomplicated malaria (Taylor, Parobek and Fairhurst, 2012). In a large multicentre genome-wide association study (GWAS), HbAS conferred 86% protection against severe malaria (Malaria Genomic Epidemiology Network, 2014). Thus, HbAS has risen to high frequencies in malaria-endemic regions as a strong selective advantage reaching a prevalence of over 40% in some parts of Africa (Mulumba and Wilson, 2015). The degree of protection conferred by HbAS on malaria exceeds any other malaria-associated polymorphisms (Malaria Genomic Epidemiology Network, 2014). Indeed, only HbAS consistently protects from uncomplicated malaria (Taylor, Parobek and Fairhurst, 2012). Thus, this mutation has the potential to be utilised to proxy malaria exposure for both severe and uncomplicated malaria.

Similar to HbAS, another haemoglobinopathy, the haemoglobin C (HbC) mutation results from a substitution of glutamine to lysine at amino acid position 6 of *HBB*. It is common in West African populations with prevalence reaching 15% (Piel *et al.*, 2013; Malaria Genomic Epidemiology Network, 2014). A meta-analysis of case-control studies showed that individuals carrying the homozygous (HbCC) and heterozygous (HbAC) mutations are 73% and 17% protected against the risk of severe malaria respectively (Taylor, Parobek and Fairhurst, 2012). Similarly, each copy of the of the HbC allele was associated with 29% protection from severe malaria (Malaria Genomic Epidemiology Network, 2014). However, the evidence is mixed on whether HbCC or HbAC protects from uncomplicated malaria or asymptomatic parasitaemia (reviewed and meta-analysed in (Taylor, Parobek and Fairhurst, 2012).

Another group of commonly reported haemoglobinopathies is the thalassaemias. Alpha (α)-thalassaemia results from mutations in alpha-globin (*HBA1* and *HBA2*) genes while beta (β)-thalassaemia results from mutations in the *HBB* gene. These mutations lead to a reduction or absence of one or more alpha- or beta- globin allele(s). For example, α -thalassemic individuals have a deletion of one or more α -globin allele(s): heterozygous ($-\alpha/\alpha$), homozygous ($-\alpha/-\alpha$ or $\alpha\alpha/--$), Haemoglobin H disease ($-\alpha/--$, a loss in three α -globin alleles), and hydrops fetalis (loss of all four α -globin alleles). β -thalassaemia results from impaired production of one β -globin allele (called β -thalassaemia minor or trait) or two β -globin alleles (called β -thalassaemia major) (Taylor, Cerami and Fairhurst, 2013).

Alpha-thalassaemia is found predominantly in sub-Saharan Africa, the Middle East, Southeast Asia, and India at a prevalence of 10-20% while β -thalassaemia is mainly prevalent in the Mediterranean and a few parts of sub-Saharan Africa (Taylor, Cerami and Fairhurst, 2013). There are conflicting findings on whether heterozygotes or homozygotes are protected from uncomplicated malaria. Compared to normal individuals, the incidence of uncomplicated malaria in α -thalassaemia homozygotes and heterozygotes was higher in Vanuatu (Williams *et al.*, 1996), but was lower in Kenya (Williams, Mwangi, Wambua, Peto, *et al.*, 2005) and Tanzania (Enevold *et al.*, 2008). However, α -thalassaemia homozygotes and heterozygotes are protected against severe malaria (Williams, Wambua, *et al.*, 2005; Taylor, Parobek and Fairhurst, 2012). The mechanisms by which haemoglobinopathies may protect from *P. falciparum* malaria are not fully elucidated. Possible mechanisms involve attenuation of erythrocytic growth of the parasite, reduced parasite endothelial cytoadherence and rosetting, as well as enhancement of innate and adaptive immunity to the parasite (reviewed in (Taylor, Cerami and Fairhurst, 2013).

Lastly, an X-linked genetic mutation associated with a deficiency in the enzyme G6PD, which plays a role in reducing oxidative stress in RBCs, has been implicated in protection from *P. falciparum* malaria. Although findings have been controversial on which G6PD allele protects from malaria, a recent meta-analysis indicated that G6PD deficiency conferred 30% protection against uncomplicated malaria in heterozygous females (Mbanefo *et al.*, 2017). Similarly, a well-powered study among Kenyan children showed that G6PD-deficient heterozygous girls were 18% protected against severe malaria but not against uncomplicated *P. falciparum* malaria or parasitaemia (Uyoga *et al.*, 2015).

1.3 Iron and malaria

In this section, I will provide a background to the relationship between iron status and malaria. I will first discuss difficulties in estimating iron deficiency in malaria endemic regions. I will then discuss how malaria may be causing iron deficiency in African children and limitations of epidemiological approaches in testing that hypothesis. I will further discuss the effects of iron status on malaria risk by reviewing the literature on iron deficiency and supplementation on malaria risk. Finally, I will discuss the association between a ferroportin coding gene mutation, *FPN* Q248H, and iron status and malaria risk.

1.3.1 Estimating the prevalence of iron deficiency in malaria-endemic areas

Determining iron status in malaria endemic countries is challenging due to the effect of malaria on iron metabolism (Aguilar *et al.*, 2012). Malaria causes a systemic maldistribution of iron into storage compartments. For example, through up-regulation of hepcidin, malaria causes blockade of iron recycling within the reticuloendothelial system as hepcidin degrades ferroportin (Howard *et al.*, 2007; Ayoya *et al.*, 2009; de Mast *et al.*, 2009; Casals-Pascual *et al.*, 2012; Atkinson *et al.*, 2015). Perhaps more importantly, malaria directly or indirectly

influences conventional iron biomarkers (discussed in Section 1.1.2). Malaria infection is associated with elevated plasma ferritin concentrations (Das, Thurnham and Das, 1997; Odunukwe *et al.*, 2000) and may also raise ferritin concentrations independently of malaria-associated inflammation (Namaste, Rohner, *et al.*, 2017). Thus, the use of ferritin concentrations to indicate iron deficiency in malaria endemic areas may underestimate the burden of iron deficiency.

Other commonly used iron biomarkers are also affected by malaria. sTfR is recommended as an alternative indicator of iron status since it is only moderately influenced by inflammation (World Health Organization and Centers for Disease Control, 2007). However, sTfR concentrations can be markedly elevated during malaria infection (Menendez *et al.*, 2001; Verhoef *et al.*, 2001; Rohner *et al.*, 2017) although some studies have found a reduction in sTfR during malaria infection (Williams *et al.*, 1999; Beesley *et al.*, 2000). ZPP concentrations are also elevated in malaria infection (Stoltzfus *et al.*, 1997, 2000). Malaria infection was associated with increased ZPP independently of inflammation in Kenyan preschool children (Teshome *et al.*, 2017) thus limiting its diagnostic utility in this population even among pregnant women (Mwangi *et al.*, 2014). Malaria may also influence transferrin saturation since destruction of RBCs during malaria infection increases serum iron (Das, Thurnham and Das, 1997). Thus, the use of conventional markers of iron status may be limited in malaria endemic areas.

Despite the influence of malaria on iron biomarkers, the presence of malaria parasitaemia is often not adjusted for when defining iron deficiency. A study in Burkinabe children (48.5% with malaria parasitaemia) showed that adjustment for malaria parasitaemia, in addition to inflammation, led to a 15.9% further absolute increase in the prevalence of iron deficiency

(Wessells and Hess, 2014). Another multicentre study including children from Africa showed that malaria was independently associated with ferritin concentrations although adjustment for malaria, in addition to inflammatory markers, only slightly increased the prevalence of iron deficiency (Namaste, Rohner, *et al.*, 2017). In that study, the prevalence of malaria ranged from 19.7% to 32.5%. It is therefore possible that the independent effects of malaria on iron status may be higher in areas where malaria is more prevalent.

Estimating iron status in malaria endemic areas is therefore difficult and a number of approaches have been proposed to account for the influences of inflammation and malaria. These include 1) regression-correction, 2) using adjustment factors, 3) using higher cutoffs, and 4) excluding individuals with inflammation (World Health Organization and Centers for Disease Control, 2007; Engle-stone *et al.*, 2013; Thurnham, Northrop-clewes and Knowles, 2015). Recently, the BRINDA (Biomarkers Reflecting Inflammation and Nutritional Determinants of Anaemia) project showed that the regression-correction approach performs better since it corrects for the linear effects of inflammation on iron biomarkers (Namaste, Aaron, *et al.*, 2017; Namaste, Rohner, *et al.*, 2017). In Chapter Three, I report predictors of iron biomarkers and use regression-correction to adjust for the effects of malaria and inflammation in estimating the ‘true’ burden of iron deficiency.

1.3.2 Malaria as a possible cause of iron deficiency

Few studies have investigated the effects of malaria infection on iron deficiency. A study in Kenyan and Gambian children observed that the prevalence of iron deficiency and iron deficiency anaemia were markedly higher at the end of a malaria season compared to the start (Atkinson *et al.*, 2014). In another study in the Kenyan highlands, interruption of malaria transmission using antimalarials and indoor residual spraying reduced the prevalence of iron

deficiency from 36% to 25% (Frosch *et al.*, 2014). However, the observational nature of these studies does not necessarily prove a causal relationship.

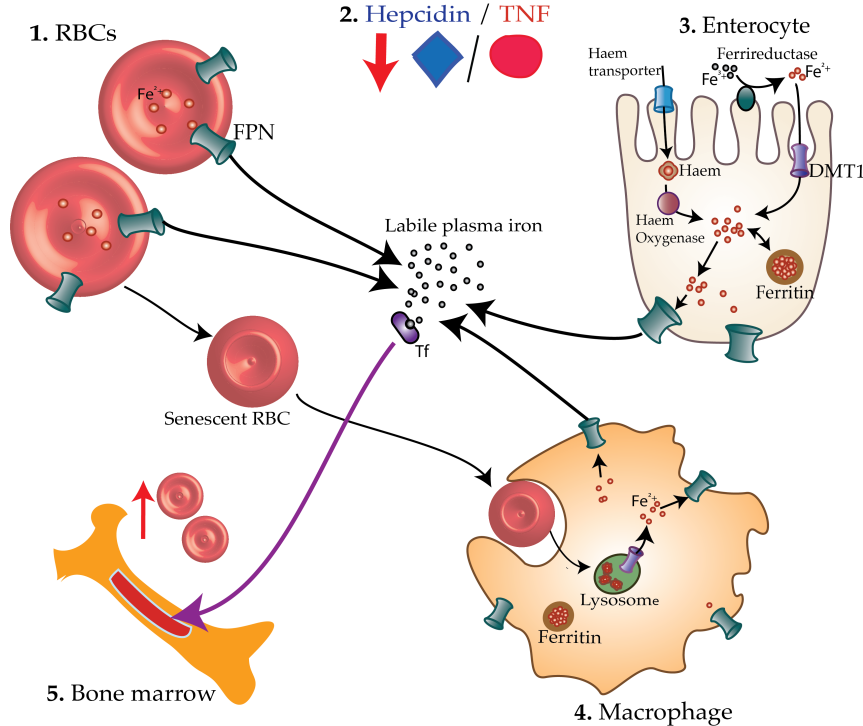
Evidence from a few previous IPT trials also suggest that malaria may cause iron deficiency. IPT trials of malaria have reported a non-significant improvement in concentrations of ferritin (Verhoef, West, Nzyuko, *et al.*, 2002; Glinz, Hurrell, Ouattara, *et al.*, 2015) and a decrease in sTfR (Desai *et al.*, 2003). Moreover, IPT of malaria was associated with a 29% reduction in the prevalence of anaemia in children following a meta-analysis of five trials (Meremikwu *et al.*, 2012). However, anaemia may not be a good indicator of iron deficiency and the reported reduction in anaemia may also be malaria-related. Although it is well known that malaria causes anaemia by the destruction of infected and uninfected RBCs, the iron released from such cells is not lost from the body but is recycled and thus it is not clear whether malaria may be causing iron deficiency.

The iron hormone hepcidin may link malaria with iron deficiency. Both clinical and asymptomatic malaria infections increase hepcidin concentrations. Clinical episodes of *P. falciparum* malaria are associated with increased hepcidin concentrations in African children (Howard *et al.*, 2007; Ayoya *et al.*, 2009; de Mast *et al.*, 2009; Casals-Pascual *et al.*, 2012; Atkinson *et al.*, 2015). Similarly, asymptomatic *P. falciparum* is associated with a doubling of hepcidin concentrations (de Mast *et al.*, 2010; Atkinson *et al.*, 2015). In Ivorian children and Beninese women, treatment of asymptomatic malaria substantially reduced hepcidin concentrations, increased iron absorption and reduced iron deficiency (Cercamondi *et al.*, 2010; Glinz, Hurrell, Righetti, *et al.*, 2015). Furthermore, the up-regulatory effects of malaria on hepcidin concentrations appear to occur both in the presence and absence of inflammation suggesting that malaria may further increase hepcidin independently of inflammation

(Atkinson *et al.*, 2015). Increased hepcidin concentrations due to frequent and chronic malaria parasitaemia in children with iron-poor diets may lead to iron deficiency since hepcidin prevents iron absorption and recycling by inhibiting the activity of ferroportin (Nemeth, Tuttle, *et al.*, 2004). However, the mechanisms through which malaria up-regulates hepcidin production are not fully elucidated, but may involve the BMP-SMAD signaling pathway (Spottiswoode *et al.*, 2017).

Malaria may also cause iron deficiency through increasing inflammatory cytokines such as tumor necrosis factor- α (TNF- α). Uncomplicated and asymptomatic malaria significantly raise TNF- α concentrations (Kwiatkowski *et al.*, 1990; Kurtzhals *et al.*, 1999). TNF- α blocks iron recycling from macrophages and inhibits erythropoiesis (Roodman *et al.*, 1987; Alvarez-Hernández *et al.*, 1989). In addition, TNF- α , independently of hepcidin, blocks intestinal iron absorption by reducing DMT1 expression (Johnson *et al.*, 2004), increasing deposition of iron into ferritin and degrading ferroportin (Laftah *et al.*, 2006). In a cohort of Gambian children, the TNF₋₃₀₈ AA genotype (which is associated with higher TNF- α transcription compared with TNF₋₃₀₈ AG and TNF₋₃₀₈ GG genotypes (Kroeger, Carville and Abraham, 1997; Wilson *et al.*, 1997)) was strongly associated with increased risk of iron deficiency and iron deficiency anaemia (Atkinson *et al.*, 2008). Interestingly, this effect was observed at the end of a malaria season when the prevalence of clinical malaria was highest (Atkinson *et al.*, 2008). Furthermore, ZPP concentrations were significantly raised in the TNF₋₃₀₈ AA genotype indicating dyserythropoiesis (Atkinson *et al.*, 2008). Figure 1.4 illustrates how malaria-induced hepcidin / TNF might contribute to iron deficiency. In the next section, I will discuss epidemiological approaches for investigating whether malaria causes iron deficiency.

A. NO MALARIA



B. MALARIA

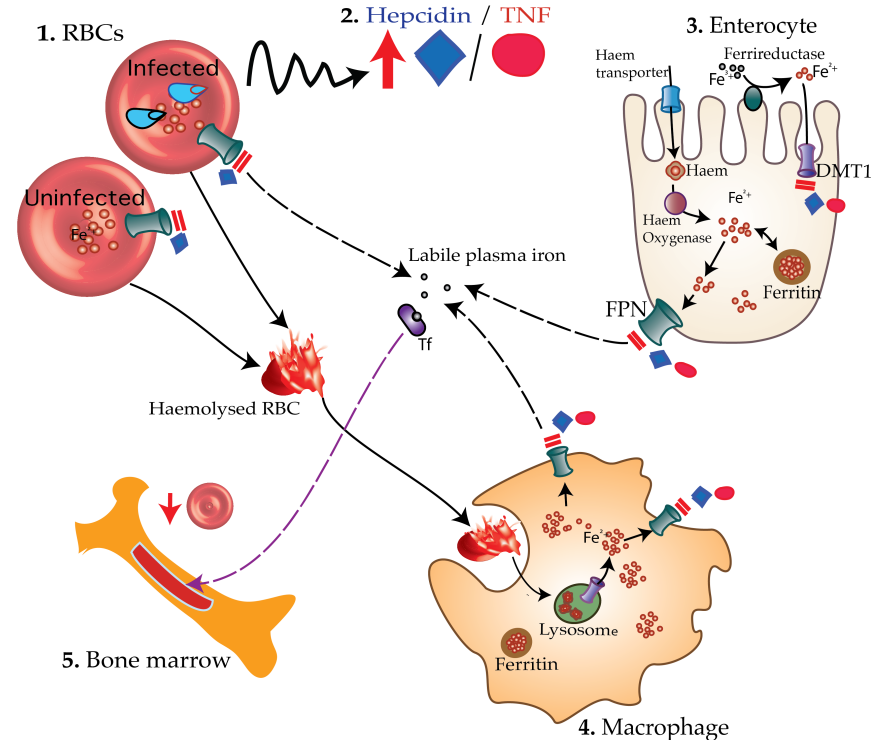


Figure 1.4. The malaria - iron deficiency hypothesis.

(A) In healthy children without malaria (A1), concentrations of hepcidin and $\text{TNF-}\alpha$ are low (A2) leading to increased absorption of iron through enterocytes (A3), reduced haemolysis of RBCs and increased recycling of iron recovered from senescent RBCs by macrophages (A4). More iron is thus available for the production of new RBCs (A5). (B) On the other hand, during malaria infection, blood-stage malaria parasites (B1) elicit increased production of hepcidin and $\text{TNF-}\alpha$ (B2), which in turn block absorption of iron through DMT1 and ferroportin (FPN) on enterocytes (B3). Hepcidin also degrades ferroportin on both infected and uninfected RBCs leading to accumulation of intracellular iron, oxidative stress, and consequently haemolysis. Haemolysed RBCs are taken up by the macrophage (B4). Hepcidin and $\text{TNF-}\alpha$ inhibit recycling of iron recovered from haemolysed RBCs back into the circulation leading to deficiency of the amount of biologically available iron. Consequently, little iron is available to produce new RBCs by the bone marrow leading to iron deficiency anaemia (B5). Tf, transferrin. I drew this diagram and it is reproduced from (Muriuki and Atkinson, 2018).

1.3.2.1 Epidemiological approaches to investigate whether malaria causes iron deficiency

It is challenging to investigate whether malaria causes iron deficiency due to potential confounders and the possibility of reverse causality. Longitudinal cohort studies following up malaria exposed and unexposed children for iron status might be one approach to investigate whether malaria causes iron deficiency. However, since individuals in malaria endemic areas are likely to be exposed to malaria repeatedly and in varying degrees over time (Snow *et al.*, 2017), it would be difficult to group them as exposed or unexposed with certainty. Individuals who are continuously exposed to malaria develop partial immunity to malaria and may be misclassified as unexposed (Bejon *et al.*, 2009). Moreover, it is difficult to specifically determine the degree of malaria exposure (Langhorne *et al.*, 2008; Olotu *et al.*, 2012). Another challenge is the fact that host iron status may also influence malaria risk making reverse causality a possibility (Nyakeriga *et al.*, 2004; Gwamaka *et al.*, 2012; Jonker *et al.*, 2012; Barffour *et al.*, 2017; Moya-Alvarez *et al.*, 2017). Pragmatic cohort studies would involve comparing iron deficiency during the course of malaria seasons although many other factors may influence iron status during that period (Atkinson *et al.*, 2014). For example, the nutritional status of children may improve during harvest seasons, which may coincide with rainy seasons (or peak malaria transmission) thereby confounding a possible effect of malaria on iron status.

Another approach would be to spatially and temporally map-out the distribution of malaria and iron deficiency. If malaria causes iron deficiency, then areas or periods of high malaria transmission would also be associated with higher prevalence of iron deficiency. Carefully gathered and mapped epidemiological data of malaria in Africa, both in space and time, are available (Noor *et al.*, 2014; Snow *et al.*, 2017). Likewise, a number of iron deficiency studies have been conducted in African children over the years although not mapped. However, this

approach is limited by the fact that levels of ferritin and other iron biomarkers are raised by malaria infection (discussed in Section 1.3.1). Thus, malaria may obscure the true picture of iron deficiency so that children in malaria regions may appear more iron replete. Social economic status may further confound the geographical distribution of malaria and iron deficiency since both are more likely to occur in the poorest communities.

Randomised controlled trials remain the gold standard study designs for investigating a causal relationship. Individuals could be randomised to receive interventions known to be effective against malaria such as antimalarials and insecticide-treated bed nets and then iron status could be assessed after a period of time. For example, children could be randomised to receive IPT of malaria followed by assessment of iron status. In a few previous trials, IPT of malaria was associated with non-significant improvement in ferritin concentrations and a decrease in sTfR suggesting modest improved iron status (Verhoef, West, Nzyuko, *et al.*, 2002; Desai *et al.*, 2003; Glinz, Hurrell, Ouattara, *et al.*, 2015). However, both ferritin and sTfR are raised during malaria infection (Das, Thurnham and Das, 1997; Phiri, Calis, Siyasiya, *et al.*, 2009; Aguilar *et al.*, 2012) making interpretation of iron status difficult and thus they may not be the best indicators of the effect of IPT on iron status. Transferrin saturation may be a good indicator of improved iron absorption while reduced ZPP concentrations may indicate improved erythrocyte iron incorporation. However, it may be expensive and difficult to justify large trials randomising children to IPT of malaria or none. Thus, in Section 1.4, I will review an approach (Mendelian randomisation) that can be utilised to study whether malaria causes iron deficiency and then test the hypothesis in Chapter Four.

1.3.3 Iron status and malaria risk

Malaria parasites require iron to grow and multiply. For example, iron restriction inhibits development of both the liver and blood stage of *Plasmodium* (Matsuzaki-moriya *et al.*, 2011; Portugal *et al.*, 2011). In a seminal study by (Murray, Murray and Murray, 1975), feeding patients after admission during famine increased malaria episodes and parasitaemia and this was associated with increased serum iron and transferrin saturation. However, the exact source of iron for parasite growth and how it acquires iron remains unclear. In this section, I will discuss possible mechanisms through which the malaria parasite acquires iron and then provide evidence of associations between iron status and supplementation on malaria risk.

1.3.3.1 Iron acquisition by malaria parasite

The metabolism of iron in the malaria parasite is complex. Just like in humans, the parasite must regulate iron to meet its iron need and avoid toxicity from iron. Possible sources of iron for the parasite include haemoglobin, intracellular ferritin, traces of iron within RBCs, transferrin bound iron and non-transferrin bound iron (NTBI) (illustrated in Figure 1.5). It is thought that the parasite consumes copious amounts of haemoglobin although that is not clear. The parasite metabolises haemoglobin to acquire amino acids and release toxic haem. The parasite sequesters the toxic haem into haemozoin thus avoiding its toxicity although some trace free haem remains and gets oxidised to generate free ROS (Chugh *et al.*, 2013). The mode of action of ACTs may involve inhibition of the parasite's ability to detoxify ROS (Chugh *et al.*, 2013).

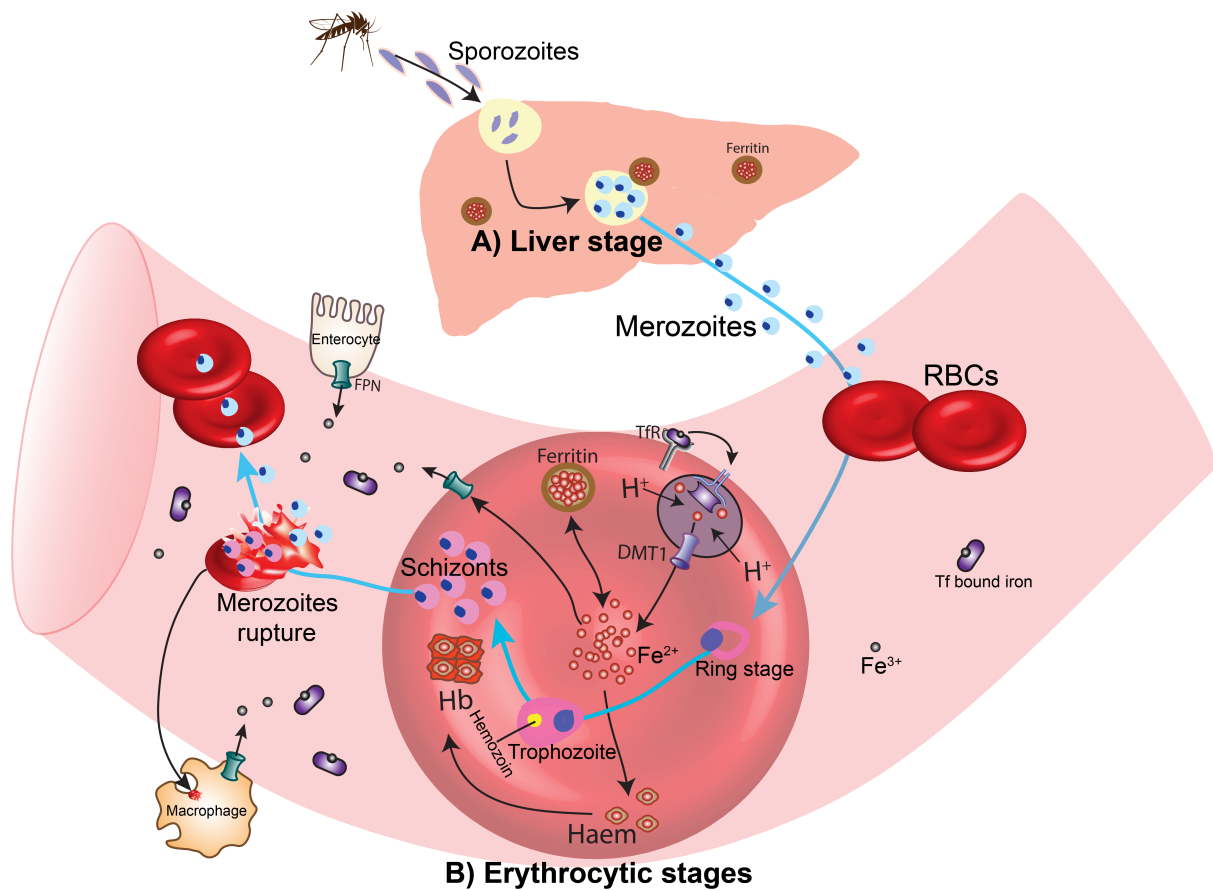


Figure 1.5. Relation between iron and *P. falciparum* stages in human.

After a bite of a malaria-infected female *Anopheles* mosquito, *Plasmodium* sporozoites infect liver cells then differentiate into merozoites before infecting red blood cells (RBCs). **A)** Presence of iron stores within hepatocytes is thought to fuel differentiation and multiplication of liver stages of *Plasmodium*. **B)** Within RBCs, the merozoites undergo a rapid intra-erythrocytic proliferation possibly fueled by the high amount of haem or stored iron within RBCs. Intracellular labile iron may also play a role in the growth of the parasite. The parasite also metabolises haem and sequesters it into haemozoin to avoid its toxicity. Once RBCs haemolyse, merozoites are released into circulation to infect new RBCs. The merozoites may also utilise non-transferrin bound iron. I drew this diagram.

Evidence suggests that the malaria parasite may utilise intracellular iron in RBCs. In a recent study, knock-out of the *FPN* gene in mice injected with *Plasmodium yoelii* YM caused an increase in parasite density and deaths compared to wild type mice (Zhang *et al.*, 2018). These results suggest that *FPN* gene knock-out may increase the labile iron pool within RBCs and promote growth of the parasite. However, it is also possible that *FPN* knock-out may have

caused iron to accumulate within RBCs leading to increased oxidative stress, haemolysis and spread of the parasite to other uninfected RBCs (Zhang *et al.*, 2018).

It is also not clear whether the malaria parasite utilises serum iron. *In vitro* parasitised RBCs have been shown to take up iron bound to transferrin (Pollack and Fleming, 1984; Clark *et al.*, 2013). However, in other studies the malaria parasite does not seem to directly utilise serum iron (Peto and Thompson, 1986; Pollack and Schnelle, 1988). Thus, it is not clear whether any perturbations to host iron (either by iron deficiency or improved iron status through supplementation) may influence the risk of malaria infection.

1.3.3.2 Iron status and malaria risk in observational studies

Previous observational studies have all shown that iron deficiency is associated with a reduced risk of clinical and severe *P. falciparum* malaria in African children (Nyakeriga *et al.*, 2004; Gwamaka *et al.*, 2012; Jonker *et al.*, 2012; Barffour *et al.*, 2017; Moya-Alvarez *et al.*, 2017). (Nyakeriga *et al.*, 2004) reported a 30% reduction in clinical malaria during a six-month follow-up of 240 Kenyan children aged 8 months to 8 years. Similarly, studies in Malawi (727 children aged 6 - 60 months with one year of follow-up) (Jonker *et al.*, 2012) and Tanzania (785 children followed-up from birth to 3 years) (Gwamaka *et al.*, 2012) reported reduced malaria risk of 45% and 23% respectively. Other more recent studies in 745 Zambian children aged 4 - 8 years followed-up for six months, and in 400 Beninese infants followed-up from birth to 1 year of age, reported an increased risk of malaria in children with high ferritin concentrations (Barffour *et al.*, 2017; Moya-Alvarez *et al.*, 2017). However, despite the wide range of iron biomarkers, these studies largely reported iron deficiency defined using ferritin only. As discussed in Sub-section 1.3.1, ferritin is substantially increased by malaria thus, the protection observed may indicate an acute phase response from malaria infection. Moreover, it

is difficult to control for reverse causality in observational studies. Thus, there is a need for more pragmatic or innovative study designs to infer causality.

1.3.3.3 Iron status and malaria risk in clinical trials

In contrast, randomised controlled trials have reported inconsistent findings for the effects of iron on malaria. Some have reported that iron supplementation does not influence risk of malaria (Menendez *et al.*, 1997), others that iron supplementation reduces malaria risk (Zlotkin *et al.*, 2013), while others that iron supplementation increases malaria risk (Sazawal *et al.*, 2006; Veenemans *et al.*, 2011; Esan *et al.*, 2013). In a large trial (24,076 children) that was conducted in Pemba, Tanzania in 2006, iron supplementation increased the risk of malaria-related events by 16% compared to the placebo group (Sazawal *et al.*, 2006). However, in a subgroup with iron measurements, this study reported a 44% reduced risk of malaria-related adverse events following iron supplementation among iron-deficient children at baseline. Contrary to the Pemba study, supplementation with iron in another large trial in Ghana in 2013 resulted in a 13% reduction in malaria incidence and a lack of evidence of increased malaria risk and hospital admissions among iron replete children at baseline (Zlotkin *et al.*, 2013). However, multi-nutrient (including iron) supplementation of children in a Tanzanian trial in 2011 increased malaria risk by 41% among children that were iron deficient at baseline but not among iron replete children (Veenemans *et al.*, 2011).

There are a number of possible explanations for the controversy of whether improving iron status through iron supplementation influences malaria risk. First, is the baseline randomisation of participants based on iron status. In the Pemba sub-study conflicting results were observed against the main study when they considered individuals with and without baseline iron deficiency (Sazawal *et al.*, 2006). Moreover, baseline iron status in the Pemba study was

indicated by ZPP (Sazawal *et al.*, 2006) while ferritin was used to indicate iron status in the 2011 Tanzanian (Veenemans *et al.*, 2011) and 2013 Ghanaian (Zlotkin *et al.*, 2013) studies. Second, is the provision of malaria interventions during the study. It is possible that the reduction in the incidence of malaria following iron supplementation in Ghana may be attributable to malaria interventions (such as ITNs and antimalarials) that were provided during the study (Zlotkin *et al.*, 2013). Third, is the administration of iron alone or in combination with other micronutrients which varied across the studies. Other factors include differences in study populations, intensity of malaria transmission, and factors relating to participants such as immunity to malaria and genetic predisposition. It is possible that immunity to malaria and carriage of haemoglobinopathies or G6PD deficiency, which protect from malaria, may confound possible causal effects of iron status on malaria risk. These conflicting findings have therefore left long-standing concerns among clinicians and policy makers regarding the safety of iron supplementation to correct iron deficiency in malaria-endemic regions such as sub-Saharan Africa (Suchdev *et al.*, 2010). In Chapter Five, I report analyses from longitudinal studies on whether a child's iron status influences their subsequent risk of malaria infection.

1.3.4 The ferroportin gene, iron deficiency and risk of malaria

Ferroportin which is encoded by the *SLC40A1* or *FPN* gene is the only known mammalian iron exporter (Donovan *et al.*, 2000). Ferroportin is highly expressed in duodenal enterocytes and macrophages where it plays a crucial role in iron absorption and recycling, respectively (Donovan *et al.*, 2005; Drakesmith, Nemeth and Ganz, 2015). A recent study showed that ferroportin is also abundant in RBCs and plays a role in reducing intracellular iron accumulation and haemolysis (Zhang *et al.*, 2018). To optimize iron homeostasis, hepcidin occludes and degrades ferroportin during iron overload (Nemeth, Tuttle, *et al.*, 2004;

Aschemeyer *et al.*, 2018). Heparidin also degrades ferroportin during infections to deny pathogens of iron.

A number of polymorphisms in the *SLC40A1* gene have been implicated in iron overloading (Mayr *et al.*, 2010). One such polymorphism is the Q248H (glutamine to histidine at position 248) mutation which occurs primarily in African populations (Beutler *et al.*, 2003; Gordeuk *et al.*, 2003). The *FPN* Q248H mutation may increase cellular iron export by rendering ferroportin partially resistant to degradation by hepcidin resulting in a 60% decrease in intracellular iron in Q248H cells (Nekhai *et al.*, 2013). Thus, in enterocytes and macrophages the mutation might increase absorption and recycling of iron. However, Q248H has also been shown to impair cellular iron export (McGregor *et al.*, 2005) or have no functional effect (Drakesmith *et al.*, 2005; Schimanski *et al.*, 2005). Moreover, previous studies with limited sample sizes, have found conflicting associations between Q248H and haemoglobin concentrations (Beutler *et al.*, 2003; Gordeuk *et al.*, 2003; McNamara, Gordeuk and MacPhail, 2005; Masaisa *et al.*, 2012; Zhang *et al.*, 2018) or with iron deficiency (Beutler *et al.*, 2003; Gordeuk *et al.*, 2003; Kasvosve *et al.*, 2005, 2010, 2015; Rivers *et al.*, 2007; Nekhai *et al.*, 2013; Cikomola *et al.*, 2017).

Since the *FPN* Q248H mutation occurs mainly in African populations, it has been hypothesised that it is positively selected to protect from malaria risk (Zhang *et al.*, 2018). A recent study showed that the Q248H mutation is associated with protection from malaria risk in Zambian children and primiparous Ghanaian women (Zhang *et al.*, 2018). In the study, children carrying Q248H had lower *P. falciparum* asexual parasites in peripheral blood compared to children with the wild type allele. Similarly, the proportion of placental *P. falciparum* was lower in Q248H carriers compared to women with wild type allele. However, the study was of very limited size: 13 Q248H children with uncomplicated malaria and 11 Q248H women with

placental malaria. Furthermore, (Zhang *et al.*, 2018) included only cases and no controls for their study of uncomplicated malaria among Zambian children and thus were unable to assess risk of malaria. They also reported contrasting results since geometric mean parasite densities in both peripheral and placental blood were higher in Q248H carriers compared to wild-type and no difference was observed in microscopy positive peripheral or placental blood (Zhang *et al.*, 2018). In Chapter Six, I report large scale genomic datasets determining whether the Q248H mutation protects from iron deficiency, anaemia and malaria risk.

1.4 Mendelian randomisation

In Section 1.3.2.1, I discussed some limitations of epidemiological approaches for determining whether malaria causes iron deficiency. In this section, I will discuss Mendelian randomisation as an approach to test the hypothesis that malaria causes iron deficiency. Mendelian randomisation utilises genetic variants as proxies for modifiable environmental exposures (or instrumental variables) to infer a causal relationship between an exposure and an outcome (Davey Smith and Ebrahim, 2003). This study design provides an alternative to randomised controlled trials since genetic variants are unlikely to be confounded by environmental factors and reverse causality is eliminated as genetic variants are allocated at conception (Evans and Davey Smith, 2015) (Figure 1.5). Mendelian randomisation also reflects a life-time of exposure, which is important since age is a critical determinant of infectious risk. This approach has been successfully employed in other disease processes and has helped to explain previous controversies (Elliott *et al.*, 2009; Voight *et al.*, 2012; Afzal *et al.*, 2014).

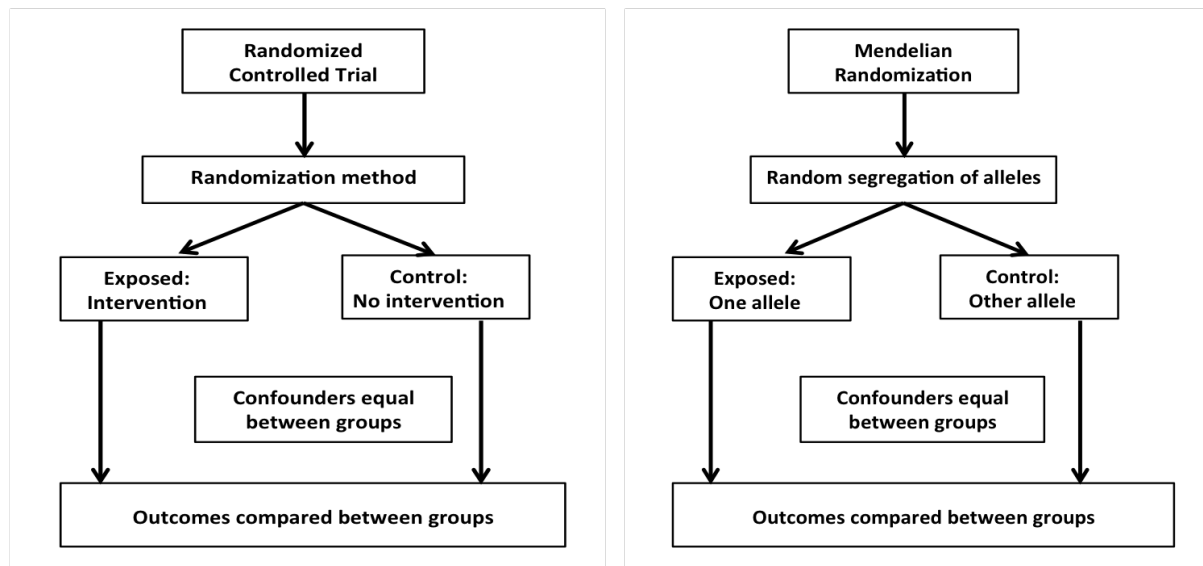


Figure 1.6. Comparison of a randomised controlled trial and Mendelian randomisation.

Adapted from (Davey Smith, Timpson and Ebrahim, 2008)

Similarly, Mendelian randomisation can be utilised to study whether malaria causes iron deficiency. There are known genetic polymorphisms that are associated with resistance to malaria such as sickle cell trait (HbAS), α -thalassaemia, and G6PD (discussed in Section 1.2.4) among others (Malaria Genomic Epidemiology Network, 2014). If malaria causes iron deficiency, then polymorphisms that protect from malaria should also be associated with reduced risk of iron deficiency. However, the polymorphisms should only influence iron status through malaria, thus the effect of the polymorphism(s) on iron status should only be observed in populations at risk of malaria, but not in malaria-free populations (i.e. there should be no pleiotropy or independent effect of the polymorphisms on iron deficiency). Additionally, each of the protective polymorphisms should influence iron deficiency in the same direction (Burgess, Butterworth and Thompson, 2013; Bowden, Davey Smith and Burgess, 2015). Figure 1.6 illustrates the conceptual Mendelian randomisation causal diagram for malaria and iron deficiency.

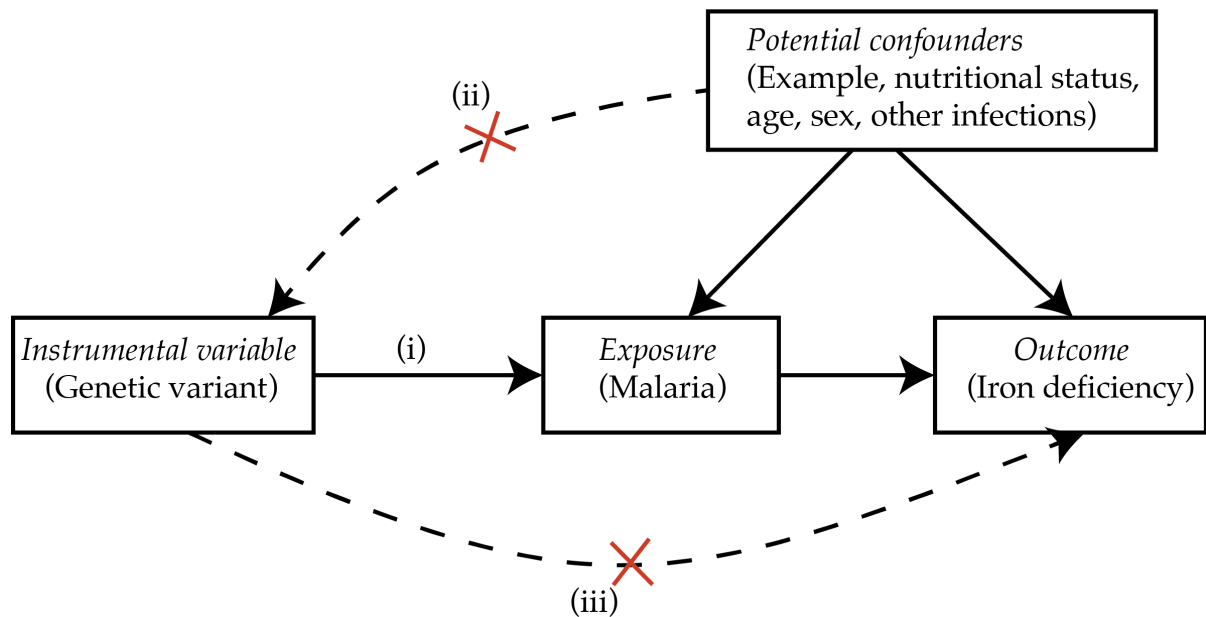


Figure 1.7. Conceptual Mendelian randomisation causal inference framework.

(i) Genetic variants reliably associated with malaria are required, for example, sickle cell trait. (ii) The genetic variant should not be associated with any measured potential confounders. (iii) The genetic variant should influence iron deficiency only through malaria exposure, i.e. the effects should not be observed in populations that are not exposed to malaria. Adapted from Sheehan *et al* (Sheehan *et al.*, 2008).

There is little and conflicting information on how carriage of haemoglobinopathies influence iron status. HbAS (n = 75) was associated with reduced frequency of iron deficiency anaemia among inhabitants of a Lebanese village in an early study (Hershko *et al.*, 1982). However, more recent small studies have reported mixed findings on the effect of sickle cell trait on iron status. For example, (Nyakeriga *et al.*, 2005) did not find a difference in iron status between Kenyan children carrying HbAS (n = 39) and normal haemoglobin (HbAA) while (Engle-stone *et al.*, 2017) found that HbAS (n = 40) was associated with increased iron stores among Cameroonian children. In children from Vanuatu, alpha-thalassaemic heterozygotes (n = 68) and homozygotes (n = 46) had increased sTfRs concentrations compared to normal controls, but there was no difference in ferritin levels (Rees *et al.*, 1998). It is possible that, in a large study, these polymorphisms may protect from iron deficiency by reducing malaria risk.

1.5 Research questions raised during this literature review

This literature review raises a number of research questions that I will address in this thesis.

These include:

1. How can we more accurately estimate the burden of iron deficiency in African children?

The challenge is that conventional markers of iron status are influenced by inflammation and malaria thereby obscuring the true picture of iron deficiency in this population. In Chapter Three, I apply adjustments for the effects of inflammation and malaria on iron biomarkers to estimate the ‘true’ burden of iron deficiency. I also test the diagnostic utility of various iron biomarkers in predicting the burden of iron deficiency.

2. Does malaria cause iron deficiency? It is difficult to answer this question in observational studies since malaria up-regulates iron markers and iron status may also influence malaria risk (i.e. reverse causality). In Chapter Four, I apply a Mendelian randomisation approach using genetic proxies for malaria to answer this question.

3. Does iron status influence malaria risk? Previously, observational studies of limited size have only looked at the effect of ferritin-defined iron deficiency on malaria. How about other markers of iron status? In Chapter Five, I investigate whether a child’s iron status influences their subsequent risk of malaria infection using a wide range of iron biomarkers in two large cohorts of children.

4. Can genetics help us determine whether iron status influences malaria risk? Since the *FPN* Q248H preserves ferroportin thereby increasing intracellular iron export, how does that influence iron status and malaria risk? In Chapter Six, I report analyses of

large datasets to determine the associations between *FPN* Q248H mutation and iron status, anaemia, and malaria. An important future question (which will not be addressed in this thesis) is to identify genetic variants that reliably alter iron status in African children, through a genome-wide association study, and use the identified variants to determine whether they are also associated with malaria risk in Mendelian randomisation analyses.

1.6 Aim and objectives of the study

Following the above questions, the general aim of this thesis is therefore to determine the causal relationship between iron deficiency and malaria. Specifically, the thesis objectives include:

1. To determine the predictors of iron biomarkers and estimate the ‘true’ burden of iron deficiency in African children,
2. To determine whether malaria is causally associated with iron deficiency,
3. To determine whether iron status is associated with malaria infection, and
4. To determine whether a mutation in the ferroportin gene (Q248H) is associated with iron status, anaemia and malaria risk.

The next chapter details the general methods used to answer the above questions. Thereafter, chapters are organised based on the above objectives. Finally, I present a general discussion detailing conclusions and future research.

CHAPTER TWO: GENERAL METHODS

In this chapter, I describe the main study populations included in this thesis, laboratory methods for measurement of iron and inflammatory biomarkers, as well as measurements for malaria parasitaemia and anthropometry. I finally highlight the definitions of key outcomes reported in Chapters Three to Six. Chapter-specific methods are described within the respective Chapters.

2.1 Study populations

Analyses in this thesis included data from 12 community-based studies, one hospital-based study, and the MalariaGEN consortium severe malaria datasets (Malaria Genomic Epidemiology Network, 2014). The community-based studies included nine from the malaria-endemic regions of sub-Saharan Africa (in Kenya, Uganda, Burkina Faso, The Gambia, Malawi, Ghana, Democratic Republic of Congo (DRC), Tanzania, and Cameroon), and two studies in non-malaria exposed cohorts of South African children and African-Americans from the Jackson Heart Study (JHS) in Mississippi, USA. The hospital-based study was conducted in Kilifi County Hospital, Kilifi, Kenya. Figure 2.1 shows the African study sites mapped against a map of the prevalence of *P. falciparum* between 2010 and 2015 as previously published by (Snow *et al.*, 2017).

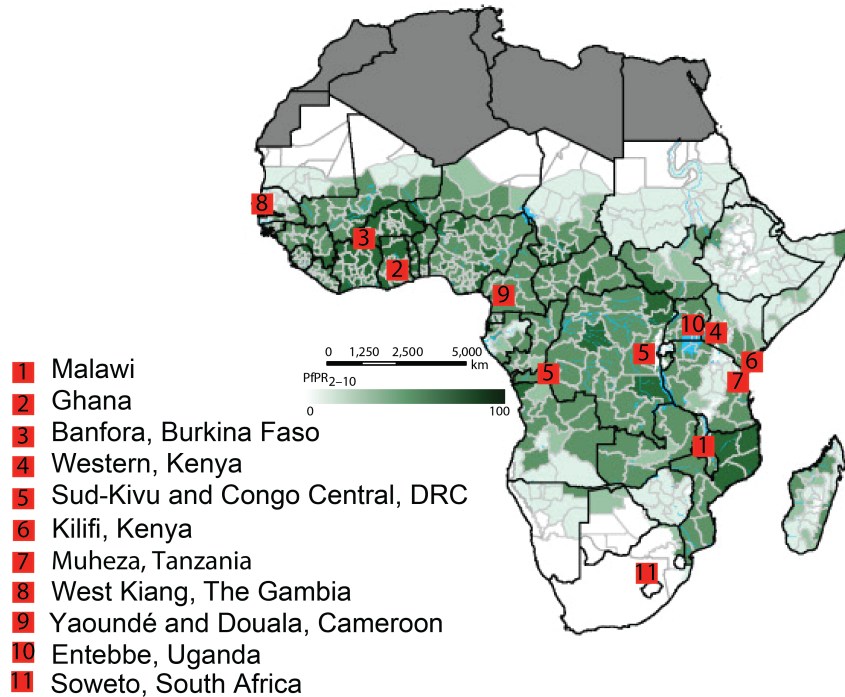


Figure 2.1. African study sites.

Studies are ordered based on the study prevalence of malaria parasitaemia. Highest in Malawi. Note, historically, there is no malaria in South Africa.

These studies can further be divided into my role in data generation and curation. I was actively involved in data generation in four community-based studies: Kilifi, Kenya; Banfora, Burkina Faso; Entebbe, Uganda; and Soweto, South Africa. In Kilifi, Kenya, I also selected, retrieved and aliquoted plasma/serum samples. In these four studies, measurements of iron and inflammatory markers were performed at the Department of Clinical Biochemistry, University of Oxford by Ms Wandia Kimita (Dr Sarah Atkinson's master's student at the KEMRI-Wellcome Trust Research Programme) and under the supervision of Dr Alireza Morovat, Consultant Clinical Biochemist (Oxford University Hospitals). I merged all the generated raw data files and checked for inconsistencies (such as samples with values below the detection limit and expected range of values from the assay manuals). In this thesis, I will refer to these four studies as the *iron_vaccgene* studies since the studies were designed in collaboration with Dr. Alexander Mentzer (University of Oxford) whose interest was in the genetics of vaccine

responses. I obtained data for the remaining study sites through collaborations with other investigators who had already measured markers of iron status and inflammation, tested for malaria, and conducted anthropometry and genotyping (Table 2.1). I then cleaned and analysed all datasets. Below, I describe the five main studies included in this thesis: the *iron_vaccgene* studies and The Gambia study. The rest of the studies (Malawi; Ghana; Western, Kenya; Sud-Kivu and Congo central, DRC; Muheza, Tanzania; Yaoundé and Douala, Cameroon; and Jackson Heart Study, USA) had permission through collaboration for the analyses of whether malaria causes iron deficiency and are described in Chapter Four. I also analysed the effect of *FPN* Q248H on severe malaria using data from the MalariaGEN consortium (Malaria Genomic Epidemiology Network, 2014) with the help of Dr Gavin Band (University of Oxford). I analysed the Kenyan MalariaGEN dataset while Dr Gavin Band analysed MalariaGEN datasets for The Gambia, Malawi and Ghana.

Table 2.1. Data from individual study sites through collaboration

| Study site | Collaborator(s) | Total sample size | Age (years) | Data |
|---------------------------------|--|-------------------|-------------|---|
| Kilifi, Kenya | Prof Bejon Dr Ndungu Prof Williams | 1484 | 0 – 8 | Age, sex, iron and inflammatory markers, malaria parasitaemia (including longitudinal data), anthropometry, sickle, α -thalassaemia, G6PD, and Q248H |
| Banfora, Burkina Faso | Prof Hill Dr Mentzer Dr Sodiomon | 348 | 0 – 2 | Age, sex, iron and inflammatory markers, malaria parasitaemia, anthropometry, sickle, α -thalassaemia, G6PD, and Q248H |
| Entebbe, Uganda | Prof Elliott | 1374 | 0 – 5 | Age, sex, iron and inflammatory markers, malaria parasitaemia (including longitudinal), anthropometry, sickle, α -thalassaemia, G6PD, and Q248H |
| Soweto, South Africa | Prof Madhi | 894 | 0 – 2 | Age, sex, iron and inflammatory markers, sickle, α -thalassaemia, G6PD, and Q248H |
| West Kiang, The Gambia | Prof Prentice Dr Atkinson | 753 | 2 – 6 | Age, sex, iron and inflammatory markers, malaria parasitaemia, anthropometry, sickle, G6PD, and Q248H |
| Malawi | Prof Suchdev | 1233 | 0 – 5 | Age, sex, iron and inflammatory markers, malaria parasitaemia, anthropometry, sickle, α -thalassaemia, and G6PD |
| Ghana | Dr Wegmüller Dr Wirth | 1159 | 0 – 5 | Age, sex, iron and inflammatory markers, malaria parasitaemia, anthropometry, sickle, and α -thalassaemia |
| Western Kenya | Prof Stewart | 488 | 1 – 2 | Age, sex, iron and inflammatory markers, malaria parasitaemia, anthropometry, sickle, and α -thalassaemia |
| Sud-Kivu and Congo central, DRC | Prof Karakochuk Prof Green | 748 | 0 – 5 | Age, sex, iron and inflammatory markers, malaria parasitaemia, anthropometry, sickle, α -thalassaemia, and G6PD |
| Muheza, Tanzania | Prof Duffy | 680 | 0 – 3 | Age, sex, iron and inflammatory markers, anthropometry, sickle, α -thalassaemia, and G6PD |
| Yaoundé and Douala, Cameroon | Prof Engle-Stone | 304 | 1 – 5 | Age, sex, iron and inflammatory markers, malaria parasitaemia, anthropometry, sickle, and α -thalassaemia |
| Jackson Heart Study, USA | Dr Raffield Dr Musani | 3457 | 21 – 95 | Age, sex, iron and inflammatory markers, anthropometry, sickle, α -thalassaemia, and G6PD |
| MalariaGEN | Prof Kwiatkowski | 11,982 | 0 – 8 | Age, sex, malaria parasitaemia, haemoglobin, haematocrit, Blantyre coma test, and Q248H |

Kilifi, Kenya: This included an ongoing rolling cohort evaluating malaria immunity in children in Kilifi, along the coast of Kenya (Bejon *et al.*, 2010). Within this cohort, children were followed-up for malaria episodes up to 8 years of age with weekly follow-ups and annual cross-sectional surveys during which anthropometry measurements, auxiliary temperature, and blood samples were taken. Malaria parasitaemia (Giemsa stained thick and thin blood films) and iron and inflammatory markers were measured from blood samples collected at a single cross-sectional survey based on the availability of plasma samples archived at -80°C. Genotyping of sickle cell (rs334), α -thalassaemia, and G6PD (rs1050828) was conducted by polymerase chain reaction (PCR) (Chong *et al.*, 2000; Waterfall and Cobb, 2001) using DNA extracted by Qiagen DNA Blood Mini Kit (Qiagen, West Sussex, United Kingdom). Q248H (rs11568350) was directly typed using Agena Bioscience SEQUENOM® Matrix-Assisted Laser Desorption-Ionization Time-of-Flight (MALDI-TOF) mass spectrometry on the iPLEX platform (Ross *et al.*, 1998).

Banfora, Burkina Faso: This was the VAC050 ME-TRAP Malaria Vaccine Trial that recruited infants between the ages of 6 and 18 months living in the Banfora region of Burkina Faso to participate in a Phase 1/2b clinical trial of the safety, immunogenicity and efficacy of a viral-vectored prime-boost liver-stage malaria vaccine (Bliss *et al.*, 2017). Samples from a total of 350 infants were then selected into the *iron_vaccgene* study based on suitability of samples for DNA extraction. Plasma samples were available from the infants at multiple time-points following receipt of the experimental vaccine. Samples from individuals taken at time points as close to the 12-month age as possible were prioritised for the *iron_vaccgene* study. Anthropometry and malaria parasitaemia (Giemsa stained thick and thin blood films) were also measured (Bliss *et al.*, 2017).

Entebbe, Uganda: This is the *Entebbe Mother and Baby Study (EMaBS)* which is a prospective birth cohort that was originally designed as a randomised controlled trial to test whether anthelmintic treatment during pregnancy and early childhood was associated with differential response to vaccination or incidence of infections including malaria (<http://emabs.lshtm.ac.uk/>) (Webb *et al.*, 2011). A total of 2507 pregnant women from Entebbe and in their second or third trimester of pregnancy were initially enrolled at Entebbe Hospital antenatal clinic. A total of 2345 livebirths from these mothers were enrolled into the EMaBS study. Blood samples were collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) at birth and at subsequent birthdays up to five years of age. Anthropometry and iron / inflammatory biomarkers were measured from a single annual visit based on the availability of stored samples. The study included longitudinal active surveillance of malaria (Giemsa stained thick and thin blood films) during fortnightly home visits and quarterly clinic visits.

South Africa: The Soweto Vaccine Response Study: Infants born in Chris Hani Baragwanath Hospital living in the Soweto region of Johannesburg, South Africa were recruited from vaccine trials (Nunes *et al.*, 2016) that are coordinated by the Respiratory and Meningeal Pathogens Unit (<http://www.rmpru.com/>). Mothers of the infants were approached if the infants had received all of their EPI vaccines up to six months of age. The infants were sampled prospectively at six months of age and at 12 months after receipt of measles vaccine at nine months. Single whole blood samples were collected in EDTA vacutainer tubes for measurement of iron and inflammatory markers and DNA extraction.

The Gambia: West Kiang study: All children aged two to six years (except those with chronic illness) were recruited from 10 rural villages in the West Kiang region of The Gambia during the start of a malaria season to initially investigate seasonal genetic effects of iron status and

haemoglobin concentrations between July and August 2001 (Atkinson *et al.*, 2006). All children had a clinical examination, anthropometric measurements, and a three-day course of mebendazole for potential hookworm infection. A blood sample was collected for complete blood count, malaria slide, DNA extraction and measurement of iron / inflammatory markers. Single nucleotide polymorphisms (SNPs) for sickle cell (rs334), G6PD (rs1050828), and Q248H (rs11568350) were directly typed using Agena Bioscience SEQUENOM® MALDI-TOF mass spectrometry on the iPLEX platform (Ross *et al.*, 1998).

2.2 Assays for iron and inflammatory markers

The assayed biomarkers of iron (ferritin, soluble transferrin receptors (sTfR), hepcidin, serum iron, transferrin, unsaturated iron binding capacity (UIBC), zinc protoporphyrin (ZPP), and haemoglobin) and inflammation (C-reactive protein (CRP) and α_1 -antichymotrypsin (ACT) are shown in Table 2.2. The Gambian hepcidin values were harmonised with the rest of the cohorts' hepcidin values (measured using high sensitive DRG kit) by converting to the old DRG hepcidin assay values and then to the new high sensitive DRG hepcidin assay values (Wray *et al.*, 2017). In Uganda, haemoglobin concentrations were adjusted for an altitude of >1000m above sea level (by subtracting 0.2g/dL) (World Health Organization, 2011a).

Table 2.2. Laboratory assays for iron and inflammatory biomarkers by site

| Site | Ferritin | Soluble transferrin receptor | Hepcidin | Serum iron | Transferrin | Unsaturated iron binding capacity | Zinc protoporphyrin | Haemoglobin/MCV/MCHC | CRP | ACT |
|------------------------|--|---|---|--|--|--|--|---|--|---|
| Kilifi, Kenya | Microparticle Enzyme Immunoassay (Abbott Architect, USA) | Human sTfR ELISA (BioVendor, Czech Republic) | DRG Hepcidin 25 [bioactive] high sensitive ELISA (DRG International, USA) | MULTIGENT iron calorimetric assay, Abbott Architect, USA | Chemiluminescent Microparticle Immunoassay (Abbott Architect, USA) | Not measured | Not measured | Coulter analyser (ACT5Diff, Beckman Coulter, USA) | MULTIGENT CRP Vario assay, (Abbott Architect, USA) | Not measured |
| Banfora, Burkina Faso | Microparticle Enzyme Immunoassay (Abbott Architect, USA) | Human sTfR ELISA (BioVendor, Czech Republic) | DRG Hepcidin 25 [bioactive] high sensitive ELISA (DRG International, USA) | MULTIGENT iron calorimetric assay, Abbott Architect, USA | Chemiluminescent Microparticle Immunoassay (Abbott Architect, USA) | Not measured | Not measured | Coulter analyser (Beckman Coulter, USA) | MULTIGENT CRP Vario assay, (Abbott Architect, USA) | Not measured |
| Entebbe, Uganda | Microparticle Enzyme Immunoassay (Abbott Architect, USA) | Human sTfR ELISA (BioVendor, Czech Republic) | DRG Hepcidin 25 [bioactive] high sensitive ELISA (DRG International, USA) | Not measured | Chemiluminescent Microparticle Immunoassay (Abbott Architect, USA) | Not measured | Not measured | Coulter analyser (Beckman Coulter, Nyon, Switzerland) | MULTIGENT CRP Vario assay, (Abbott Architect, USA) | Not measured |
| Soweto, South Africa | Microparticle Enzyme Immunoassay (Abbott Architect, USA) | Human sTfR ELISA (BioVendor, Czech Republic) | DRG Hepcidin 25 [bioactive] high sensitive ELISA (DRG International, USA) | Not measured | Chemiluminescent Microparticle Immunoassay (Abbott Architect, USA) | Not measured | Not measured | Not measured | MULTIGENT CRP Vario assay, (Abbott Architect, USA) | Not measured |
| West Kiang, The Gambia | Microparticle Enzyme Immunoassay (Abbott Architect, USA) | Quantikine sTfR ELISA kit, (R&D Systems, USA) | Hepcidin-25 [human] Enzyme Immunoassay Kit (Bachem, Switzerland) | Ferrozine-based photometry and colorimetry analyser (Hitachi 911, Hitachi, Tokyo, Japan) | Not measured | Ferrozine-based photometry and colorimetry analyser (Hitachi 911, Hitachi, Tokyo, Japan) | Aviv Biomedical Hematoflurometer (within 24 hours of collection) | Medonic CA 530 Oden 16 Haemoglobinometer | Not measured | Immunoturbidimetry, Cobas Mira Plus Bio-analyser, Roche |

2.3 Genotyping in the *iron_vaccgene* studies

This section describes genotyping of sickle cell (rs334), the *FPN* Q248H mutation (rs11568350) and G6PD (rs1050828) in the *iron_vaccgene* studies except Kilifi, Kenya where typing was directly performed using PCR or Agena Bioscience SEQUENOM® MALDI-TOF mass spectrometry. I extracted the genetic variants for Ugandan, Burkinabe, and South African children from whole-genome typed data. Genotyping was performed using the Illumina HumanOmni 2.5M-8 ('octo') BeadChip array version 1.1 (Illumina Inc., San Diego, USA), at the Genotyping Core facilities at the Wellcome Trust Sanger Institute. Genotypes were called from intensities using Illuminus and GenCall clustering algorithms in GenomeStudio (Illumina Inc., San Diego, USA) incorporating data from pre-determined genotypes.

I received genotype files in binary PLINK format from Dr Alexander Mentzer. I performed quality control for samples and single nucleotide polymorphisms (SNPs) separately for each cohort (using identical steps) and using SNPs mapped to Human Genome Build 37. Dr Alex Mentzer provided files generated by the Dr Will Rayner pipeline that ensures that Illumina SNPs correctly map on human Build 37. Using the strand files (<http://www.well.ox.ac.uk/~wrayner/strand/>), I removed low quality variants (those that mapped to multiple regions within the human genome or did not map to any region) and duplicate SNPs.

I then performed quality control (Anderson *et al.*, 2010). I used PLINK (version 1.9) (<http://zzz.bwh.harvard.edu/plink/>) to calculate and filter samples with a call rate of <97% and heterozygosity outside three-standard deviations around the mean. I compared reported and genotyped sex using PLINK and removed samples with discordance using default F values of <0.2 for males and >0.8 for females. I then removed SNPs with a call rate of <97%, those in

high ($r^2 > 0.2$) linkage disequilibrium (LD, calculated using a sliding window size of 50 SNPs sliding 5 SNPs sequentially) and those with minor allele frequency $< 1\%$. I calculated identity-by-descent (IBD) to identify duplicate or related individuals and removed samples with IBD > 0.9 . I finally used PLINK to calculate scores for deviation from Hardy-Weinberg equilibrium (HWE) using individuals with IBD < 0.05 and excluded SNPs with HWE P value $< 10^{-8}$.

To impute missing genotypes, I uploaded the quality controlled genotypes on Haplotype Reference Consortium (HRC) reference panel (<http://www.haplotype-reference-consortium.org/data-access>) (Haplotype-Reference-Consortium, 2016). This reference panel is hosted in the Sanger Institute or University of Michigan servers and is not publicly available as some of the cohorts do not have consent for public release. Thus, imputation is performed remotely. The HRC reference panel consists of a number of screened cohorts including the 1000 genomes (<http://www.haplotype-reference-consortium.org/participating-cohorts>). Using the imputed data, I finally extracted the variants coding sickle cell trait (rs334), the *FPN* Q248H mutation (rs11568350) and G6PD (rs1050828) for each of the three *iron_vaccgene* studies.

2.4 Definitions

Inflammation was defined as CRP $> 5\text{mg/L}$ or ACT $> 0.6\text{g/L}$ or AGP $> 1\text{g/L}$ (World Health Organization and Centers for Disease Control, 2007). Iron deficiency was defined as plasma ferritin $< 12\mu\text{g/L}$ or $< 30\mu\text{g/L}$ in the presence of inflammation in children < 5 years or $< 15\mu\text{g/L}$ in children ≥ 5 years (World Health Organization, 2011b). Anaemia was defined as haemoglobin $< 11\text{g/dL}$ in children aged < 5 years, or haemoglobin $< 11.5\text{g/dL}$ in children ≥ 5 years (World Health Organization, 2001). Iron deficiency anaemia was defined as presence of iron deficiency and anaemia (World Health Organization, 2001). Transferrin saturation was calculated as (serum iron ($\mu\text{mol/L}$)/transferrin (g/L) $\times 25.1$) $\times 100$ or as (serum iron/ UIBC +

serum iron) x 100 (Yamanishi *et al.*, 2003). Malaria parasitaemia was defined as asexual *P. falciparum* parasite positive. Stunting was defined as height-for-age z-scores <-2, underweight as weight-for-age z-score <-2, and wasting as height-for-weight z-score <-2 (World Health Organization, 2006).

2.5 Ethical Approvals

For the *iron_vaccine* studies and The Gambia, individual study site ethical approvals were obtained. For Kilifi, Kenya (by the Scientific Ethics Review Unit of the Kenya Medical Research Institute (KEMRI/SERU/CGMR-C/046/3257/2983)); Entebbe, Uganda (locally by the Uganda Virus Research Institute (GC/127/12/07/32) and Uganda National Council for Science and Technology (MV625), and in the UK by the London School of Hygiene and Tropical Medicine (A340) and Oxford Tropical Research (OTR) (39-12, 42-14 and 37-15) Ethics Committees); Banfora, Burkina Faso (by Ministere de la Recherche Scientifique et de l'Innovation in Burkina Faso (2014-12-151) and the OTR Ethics Committees (41-12)); Soweto, South Africa (by the University of Witwatersrand Human Research (M130714) and the OTR Ethics Committees (1042-13 and 42-14)); and West Kiang, The Gambia (by the Gambian Government / Medical Research Council Ethics Committee (874/830)). For the other seven study sites, individual study data transfer agreements were signed with the responsible study/institution's principal investigator (PI) and the KEMRI-Wellcome Trust Research Programme study PI, Dr Sarah Atkinson. These other studies had ethical approval to share data for further secondary analyses presented in this thesis.

CHAPTER THREE: EPIDEMIOLOGY OF IRON DEFICIENCY IN AFRICAN CHILDREN

The material in this chapter forms the basis of a published manuscript in *BMC Medicine* entitled “*Estimating the burden of iron deficiency in African children*”, in which I was the first author.

3.1 Abstract

Background: For effective nutritional iron interventions, accurate prevalence estimates of iron deficiency are important. However, iron deficiency may be commonly underestimated in Africa since most measures of iron status are altered by inflammation and infections such as malaria. I therefore aimed to describe predictors of iron biomarkers and estimate the burden of iron deficiency in African children.

Methods: I used data on iron and inflammatory biomarkers in 4,853 children aged 0–8 years from community-based cohorts in the *iron_vaccgene* studies and The Gambia. I described iron status and its relationship with age, sex, malaria parasitaemia, inflammation, stunting, underweight, and wasting. I used regression modelling, correcting ferritin for inflammation and malaria, to estimate the prevalence of iron deficiency and compared with the WHO-defined prevalence. I further investigated the utility of other iron biomarkers in predicting the regression-corrected iron deficiency.

Results: The prevalence of iron deficiency was highest at one year of age and in male children. Stunting and underweight were associated with anaemia and iron deficient erythropoiesis. Inflammation and malaria parasitaemia were associated with all iron biomarkers, although transferrin saturation (TSAT) was least affected. Overall prevalence of ID was 52% using an inflammation and malaria regression-corrected estimate of ID, compared to a WHO-defined estimate of 34%. This WHO unidentified burden of iron deficiency increased with age and was highest in countries with high prevalence of inflammation and malaria, where up to a quarter

of iron-deficient children were misclassified as iron replete. TSAT < 11% most closely predicted the prevalence of iron deficiency according to the regression-correction.

Conclusions: The prevalence of iron deficiency is underestimated in African children when defined using WHO guidelines, especially in malaria-endemic populations and the use of TSAT may provide a more accurate approach. Further research is needed to identify the most accurate measures for determining the prevalence of iron deficiency in sub-Saharan Africa.

3.2 Introduction

Despite the detrimental effects of iron deficiency on child development (McCann and Ames, 2007), the true burden of iron deficiency in African children remains largely unknown because inflammation and infections such as malaria influence biomarkers of iron deficiency (reviewed in Chapter One). To enable planning, monitoring and targeting of effective nutritional iron interventions, reliable and accurate estimates of the prevalence of iron deficiency are necessary. However, staining of bone marrow aspirate for iron which is the gold standard method for estimating iron status, is invasive and therefore impractical in population surveys. In areas with high burden of infectious diseases, the World Health Organization (WHO) defines iron deficiency using low ferritin concentrations (<12 μ g/L in children <5 years or <15 μ g/L in children \geq 5 years), with an arbitrarily higher cut-off of ferritin (<30 μ g/L) in children <5 years with inflammation (World Health Organization and Centers for Disease Control, 2007; World Health Organization, 2011b). Ferritin is a sensitive marker of body iron stores and has standardised laboratory assays and established cut-offs. However, both inflammation and malaria independently influence synthesis of ferritin (Feelders *et al.*, 1998; Namaste, Rohner, *et al.*, 2017). I therefore hypothesised that the WHO definition of iron deficiency may underestimate the prevalence of iron deficiency in African children that are exposed to high burdens of inflammation and malaria.

Alternative markers of iron status also have limitations. Soluble transferrin receptor (sTfR) is only mildly increased during the inflammatory response (Mast *et al.*, 1998), but its utility is complicated in African populations since it is upregulated by malaria, even in asymptomatic infection (Menendez *et al.*, 2001; Verhoef *et al.*, 2001; Rohner *et al.*, 2017). Moreover, there are no standardised sTfR reference assays. Other iron biomarkers may also be confounded by the effects of inflammation or malaria (World Health Organization and Centers for Disease Control, 2007). Estimating iron status in African children is therefore challenging, although a number of approaches have been proposed to account for the effects of inflammation and malaria including using higher ferritin cut-offs, or excluding, individuals with raised inflammatory markers (Suchdev *et al.*, 2017). A regression-correction approach, which accounts for the linear effects of inflammatory markers and / or malaria on iron biomarkers, as proposed by the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anaemia (BRINDA) project, appears to reflect iron status more accurately (Namaste, Aaron, *et al.*, 2017; Namaste, Rohner, *et al.*, 2017).

In this chapter, I describe the relationship between markers of iron status and age, sex, inflammation, malaria parasitaemia, stunting, underweight and wasting. I then utilised the regression-correction approach proposed by BRINDA (Namaste, Aaron, *et al.*, 2017; Namaste, Rohner, *et al.*, 2017) to predict what the ferritin level would have been in the absence of inflammation and malaria, and then used these predicted values to estimate the prevalence of iron deficiency in African children. I then compared the regression-corrected prevalence of iron deficiency to the prevalence of WHO-defined iron deficiency. Finally, I assessed the diagnostic utility of various iron markers in predicting the regression-corrected iron deficiency as a gold standard.

3.3 Methods

3.3.1 Study population

To determine the burden of iron deficiency, I included children from the *iron_vaccgene* (Kilifi, Kenya; Entebbe, Uganda; Banfora, Burkina Faso; and Soweto, South Africa) and the West Kiang, The Gambia community-based cohorts which are described in Chapter Two Section 2.1. In these studies, measures of iron and inflammatory markers and anthropometric indices were available as described in Chapter Two Section 2.1.

3.3.2 Statistical analysis

I conducted all analyses using STATA 13.0 (StataCorp., College Station, TX). I natural (Ln)-transformed biomarkers (except transferrin and haemoglobin) to normalise their distributions. I used two-tailed Student's *t*-tests for differences in biomarker means between age groups. I fitted both univariable and multivariable linear regression models to determine the associations between iron biomarkers and age, sex, inflammation, malaria and nutritional status (indicated by stunting, underweight and wasting). I excluded Gambian sTfR, transferrin saturation (TSAT), body iron stores (BIS) and ferritin index in pooled analyses since different assays were used in this population meaning that values were not directly comparable with those from the other cohorts (Table 2.2 in Chapter Two). All p-values reflect two-tailed tests and a p-value of <0.05 was considered significant.

3.3.2.1 Regression-correction

Following analyses of predictors of iron status, I then estimated the prevalence of iron deficiency by adjusting for the effects of inflammation and malaria on ferritin levels using a regression-correction approach as developed by BRINDA (Namaste, Aaron, *et al.*, 2017; Namaste, Rohner, *et al.*, 2017). I used these estimates as the gold standard. The regression-

correction approach followed a three-step process. In the first step, I defined internal reference values for inflammatory markers (CRP or ACT) as the 10th percentile. To avoid overcorrection for very low levels of inflammatory markers only participants with CRP or ACT values above the 10th percentile (0.2mg/L and 0.3g/L for unlogged CRP and ACT respectively) had their ferritin values subtracted from observed values in equations (1) to (3) below (Namaste, Rohner, *et al.*, 2017). In the second step, I applied univariable linear regression models to the full dataset, with ferritin as the dependent variable, to estimate regression coefficients for the crude association between inflammatory marker level and ferritin (β_1), and for the crude association between malaria and ferritin (β_2), and applied multivariable linear regression to estimate adjusted regression coefficients for associations between inflammatory marker level and ferritin (β_3) and between malaria parasitaemia and ferritin (β_4). In the third step, I used the regression coefficients estimated in step 2 to calculate adjusted ferritin values using equation (1), (2) or (3). For the purposes of comparison, I applied equation (1) to adjust for the inflammatory marker only, equation (2) for malaria parasitaemia only, and equation (3) for both inflammatory marker and malaria parasitaemia. I ln-transformed ferritin and inflammatory markers before applying them in the equations.

$$Ferritin_{adjusted1} = Ferritin_{unadjusted} - \beta_1(CRP \text{ or } ACT_{obs} - CRP \text{ or } ACT_{ref}) \quad (1)$$

$$Ferritin_{adjusted2} = Ferritin_{unadjusted} - \beta_2 malaria \quad (2)$$

$$Ferritin_{adjusted3} = Ferritin_{unadjusted} - \beta_3(CRP \text{ or } ACT_{obs} - CRP \text{ or } ACT_{ref}) - \beta_4 malaria \quad (3)$$

where ‘obs’ is the observed value and ‘ref’ is the reference value.

I then defined iron deficiency using the regression-corrected unlogged ferritin (i.e. adjusted for the effects of inflammation and malaria) using the same thresholds that were applied to the uncorrected ferritin levels in the WHO recommendations (i.e. ferritin <12 μ g/L in children <5

years or $<15\mu\text{g/L}$ in children aged ≥ 5 years (World Health Organization, 2011b)) and compared changes in prevalence of iron deficiency using McNemar's chi-square test of consistency. In further models, I additionally corrected ferritin levels for age, sex and underweight. I also applied regression-correction for inflammation and malaria to other markers of iron status including sTfR, hepcidin, BIS, ferritin index, and zinc protoporphyrin (ZPP). I then tested the diagnostic utility of the uncorrected biomarkers in predicting iron deficiency regression-corrected for inflammation and malaria as the 'gold standard'. I used receiver operating characteristics curves (ROCs) analyses using regression-corrected iron deficiency as a binary classifier for identifying the optimal cut-off values of the continuous iron biomarkers. I defined the optimal cut-off value as a point on the curve where the Youden index (sensitivity + specificity-1) is maximum (Youden, 1950).

3.4 Results

3.4.1 Characteristics of study participants

A total of 4,853 children, 1484 Kenyan, 1374 Ugandan, 348 Burkinabe, 894 South African, and 753 Gambian children aged between birth and eight years were included in the analyses. Table 3.1 shows the characteristics of study participants in the five African cohorts. The prevalence of malnutrition (as indicated by stunting, underweight and wasting) was highest in Kenya and lowest in Uganda (Table 3.1). Anthropometry data were not available for South Africa. The prevalence of malaria parasitaemia was highest in Kenya (21.9%) and Burkina Faso (20.6%) and lowest in Uganda (6.8%). Similarly, the prevalence of inflammation was high in Burkina Faso (33.9%) and Kenya (27.3%), but lower in South Africa (17.6%) and in The Gambia (14.9%).

Table 3.1. Characteristics of study participants by cohort

| | Kenya, n=1484 | | Uganda, n=1374 | | Burkina Faso, n=348 | | South Africa, n=894 | | The Gambia, n=753 | | Pooled, n=4853 | |
|---------------------------------|---------------|------|----------------|------|---------------------|------|---------------------|------|-------------------|------|----------------|------|
| | n/total | % | n/total | % | n/total | % | n/total | % | n/total | % | n/total | % |
| Age in years | | | | | | | | | | | | |
| < 1 | 321/1484 | 21.6 | 26/1374 | 1.9 | 21/348 | 6.0 | 475/894 | 53.1 | 0/753 | 0.0 | 843/4853 | 17.4 |
| 1 - <2 | 597/1484 | 40.2 | 459/1374 | 33.4 | 185/348 | 53.2 | 418/894 | 46.8 | 16/753 | 2.1 | 1675/4853 | 34.5 |
| 2 - <3 | 170/1484 | 11.5 | 622/1374 | 45.3 | 142/348 | 40.8 | 1/894 | 0.1 | 188/753 | 25.0 | 1123/4853 | 23.1 |
| 3 - <4 | 159/1484 | 10.7 | 176/1374 | 12.8 | 0 | 0.0 | 0 | 0.0 | 201/753 | 26.7 | 536/4853 | 11.0 |
| 4 - 8 | 237/1484 | 16.0 | 91/1374 | 6.6 | 0 | 0.0 | 0 | 0.0 | 348/753 | 46.2 | 676/4853 | 13.9 |
| Sex: Females | 726/1484 | 48.9 | 678/1374 | 49.3 | 173/348 | 49.7 | 449/894 | 50.2 | 331/753 | 46.8 | 2378/4853 | 49.0 |
| Nutritional status ¹ | | | | | | | | | | | | |
| Stunting | 117/240 | 48.8 | 211/1355 | 15.6 | 109/325 | 33.5 | n/a | n/a | 215/592 | 36.3 | 652/2512 | 26.0 |
| Underweight | 114/429 | 26.6 | 113/1368 | 8.3 | 60/327 | 18.4 | n/a | n/a | 150/593 | 25.3 | 437/2717 | 16.1 |
| Wasting | 27/237 | 11.4 | 67/1353 | 5.0 | 22/325 | 6.8 | n/a | n/a | 51/592 | 8.6 | 167/2507 | 6.7 |
| Malaria ² | 262/1199 | 21.9 | 92/1353 | 6.8 | 66/321 | 20.6 | n/a | n/a | 84/751 | 11.2 | 504/4518 | 11.2 |
| Inflammation ³ | 392/1437 | 27.3 | 316/1337 | 23.6 | 112/330 | 33.9 | 157/894 | 17.6 | 112/753 | 14.9 | 1089/4751 | 22.9 |

n/a, not available

Median (IQR) for age in years was as follows: Kenya 1.7 (1.1, 3.1); Uganda 2.0 (2.0, 3.0); Burkina Faso 1.9 (1.6, 2.2); South Africa 1.0 (1.0, 1.0); The Gambia 3.8 (2.9, 4.9); and Pooled 2.0 (1.0, 3.0)

¹Stunting was defined as height-for-age z-score < -2, underweight as weight for age z-score < -2, and wasting as weight-for-height z-score < -2. These data were not available in the South African cohort.

²Malaria was defined as *P. falciparum* parasitaemia at any parasite density.

³Inflammation was defined as C-reactive protein > 5mg/L or α 1-antichymotrypsin > 0.6 (in The Gambia).

3.4.2 Distribution of iron status and anaemia

Table 3.2 shows the prevalence of WHO-defined iron deficiency and anaemia, and concentrations of the individual iron biomarkers by study cohort. Based on the WHO recommended definition, the prevalence of iron deficiency was highest in South African children (41.9%), lowest in The Gambia (21.7%) and affected approximately a third of children in each of Kenya (35.4%), Uganda (34.6%) and Burkina Faso (35.5%). Anaemia was present in 87.0% of children in Burkina Faso, 70.0% in Kenya, 60.1% in The Gambia and 49.7% in Uganda, while haemoglobin was not measured in South Africa. Geometric means and 95% confidence intervals of the various iron biomarkers are shown in Table 3.2.

Table 3.2. Distribution of iron status and anaemia by study cohort

| Category | Kenya | | | Uganda | | | Burkina Faso | | | South Africa | | | The Gambia | | | Pooled ¹ | | |
|--------------------------------------|----------|------|------------|----------|------|------------|--------------|------|------------|--------------|------|------------|------------|------|------------|---------------------|------|------------|
| | n/total | % | 95% CI | n/total | % | 95% CI | n/total | % | 95% CI | n/total | % | 95% CI | n/total | % | 95% CI | n/total | % | 95% CI |
| Iron deficiency ² | 499/1408 | 35.4 | 32.9, 37.9 | 438/1267 | 34.6 | 31.9, 37.2 | 115/324 | 35.5 | 30.3, 40.7 | 375/894 | 41.9 | 38.7, 45.2 | 163/752 | 21.7 | 18.7, 24.6 | 1590/4645 | 34.2 | 32.9, 35.6 |
| Anaemia ³ | 609/870 | 70.0 | 66.9, 73.1 | 652/1312 | 49.7 | 47.0, 52.4 | 288/331 | 87.0 | 83.4, 90.6 | n/a | n/a | n/a | 448/746 | 60.1 | 56.5, 63.6 | 1997/3259 | 61.3 | 59.6, 62.9 |
| Iron deficiency anaemia ⁴ | 207/833 | 24.8 | 21.9, 27.8 | 255/1209 | 21.1 | 18.8, 19.8 | 96/309 | 31.1 | 25.9, 36.3 | n/a | n/a | n/a | 123/745 | 16.5 | 13.8, 19.2 | 681/3096 | 22.0 | 20.5, 23.5 |
| Biomarker | n | Mean | 95% CI | n | Mean | 95% CI | n | Mean | 95% CI | n | Mean | 95% CI | n | Mean | 95% CI | n | Mean | 95% CI |
| Haemoglobin, g/dL | 870 | 10.2 | 10.1, 10.3 | 1312 | 9.1 | 9.0, 9.2 | 331 | 9.6 | 9.4, 9.7 | n/a | n/a | n/a | 746 | 10.7 | 10.6, 10.8 | 3259 | 10.5 | 10.5, 10.6 |
| Ferritin, μ g/L | 1408 | 21.8 | 20.6, 23.1 | 1267 | 20.8 | 19.6, 22.0 | 324 | 22.2 | 19.7, 24.9 | 894 | 14.9 | 14.0, 15.9 | 752 | 25.1 | 23.6, 26.8 | 4645 | 20.5 | 19.9, 21.1 |
| sTfR, mg/L | 1467 | 17.9 | 17.5, 18.3 | 1343 | 6.7 | 6.5, 7.0 | 342 | 17.7 | 16.7, 18.7 | 893 | 11.2 | 10.9, 11.5 | 661 | 3.4 | 3.4, 3.5 | 4045 | 11.7 | 11.4, 11.9 |
| Hepcidin, μ g/L | 1373 | 5.8 | 5.4, 6.2 | 1333 | 6.8 | 6.4, 7.2 | 309 | 5.3 | 4.6, 6.3 | 878 | 7.7 | 7.1, 8.4 | 709 | 6.0 | 5.3, 6.7 | 4602 | 6.4 | 6.2, 6.6 |
| BIS, mg/kg ⁵ | 1393 | -0.8 | -1.0, -0.5 | 1241 | 2.5 | 2.2, 2.8 | 322 | -0.6 | -1.1, -0.1 | 893 | -0.4 | -0.7, -0.2 | 660 | 5.7 | 5.4, 6.0 | 3849 | 0.4 | 0.2, 0.5 |
| Ferritin index ⁶ | 1389 | 14.4 | 13.9, 14.9 | 1234 | 5.5 | 5.3, 5.8 | 322 | 13.9 | 12.8, 15.1 | 881 | 10.1 | 9.7, 10.6 | 660 | 2.6 | 2.5, 2.7 | 3826 | 9.7 | 9.5, 10.0 |
| Serum iron, μ mol/L | 1428 | 6.5 | 6.3, 6.7 | n/a | n/a | n/a | 337 | 6.0 | 5.7, 6.4 | n/a | n/a | n/a | 737 | 8.6 | 8.3, 8.9 | 1765 | 6.4 | 6.2, 6.6 |
| Transferrin, g/L | 1409 | 2.8 | 2.7, 2.8 | 1333 | 2.8 | 2.6, 2.7 | 327 | 2.7 | 2.6, 2.8 | 894 | 2.7 | 2.7, 2.8 | n/a | n/a | n/a | 3963 | 2.8 | 2.7, 2.8 |
| TSAT, % ⁷ | 1386 | 9.4 | 9.0, 9.8 | n/a | n/a | n/a | 325 | 8.9 | 8.3, 9.7 | n/a | n/a | n/a | 734 | 12.8 | 12.3, 13.3 | 1711 | 9.3 | 9.0, 9.6 |
| ZPP, μ mol/mol haem | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | n/a | 751 | 85.9 | 82.4, 89.5 | 751 | 85.9 | 82.4, 89.5 |
| MCV, fL | 499 | 65.7 | 65.0, 66.5 | 1305 | 71.0 | 70.6, 71.5 | n/a | n/a | n/a | n/a | n/a | n/a | 741 | 75.6 | 75.1, 76.1 | 2545 | 71.2 | 70.9, 71.6 |

BIS, body iron store; sTfR; n/a, not available; soluble transferrin receptors; TSAT, transferrin saturation; ZPP, Zinc protoporphyrin; MCV, mean corpuscular volume

The mean values are geometric means except for BIS, transferrin, haemoglobin and MCV which are arithmetic means

¹Pooled for sTfR, BIS, ferritin index, iron, transferrin and TSAT excludes The Gambia since markers were measured using different assays compared to the other cohorts.

²Iron deficiency was defined by WHO definition as ferritin < 12 μ g/L or <30 μ g/L in presence of inflammation (C-reactive protein > 5mg/L or α 1-antichymotrypsin > 0.6g/L) in children < 5 years or <15 μ g/L in children \geq 5 years (World Health Organization and Centers for Disease Control, 2007).

³Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 5 years or haemoglobin < 11.5 g/dL in children above 5 years (World Health Organization, 2001). Missing in South Africa.

⁴Iron deficiency anaemia defined as iron deficiency and anaemia (World Health Organization, 2001). Haemoglobin was missing in South Africa.

⁵Body iron store was calculated using the ratio of soluble transferrin receptors and ferritin concentrations, $-(\log_{10}((sTfR \times 1000)/ferritin) - 2.8229)/0.1207$ (Cook, Flowers and Skikne, 2003).

⁶Ferritin index was defined as $sTfR/\log_{10} ferritin$ (Phiri, Calis, Siyasiya, *et al.*, 2009).

⁷TSAT, transferrin saturation was calculated as $((Iron (\mu mol/L)/Transferrin (g/L) \times 25.1) \times 100)$ except in The Gambia where it was calculated using iron and unsaturated iron binding capacity (Yamanishi *et al.*, 2003). Missing in Uganda and South Africa. Transferrin missing in The Gambia. Iron available in Kenya, Burkina Faso and The Gambia only.

3.4.3 Age and sex differences in iron status

Concentrations of ferritin, hepcidin, BIS and TSAT decreased during the first year of life and increased thereafter, suggesting that iron deficiency is most prevalent at about one year of age (Figure 3.1). Male infants were more iron deficient than female infants for each of the different measures of iron status. These sex-specific differences were not observed beyond three years of age. Haemoglobin concentrations did not differ by sex although there was a trend towards higher haemoglobin concentrations in females.

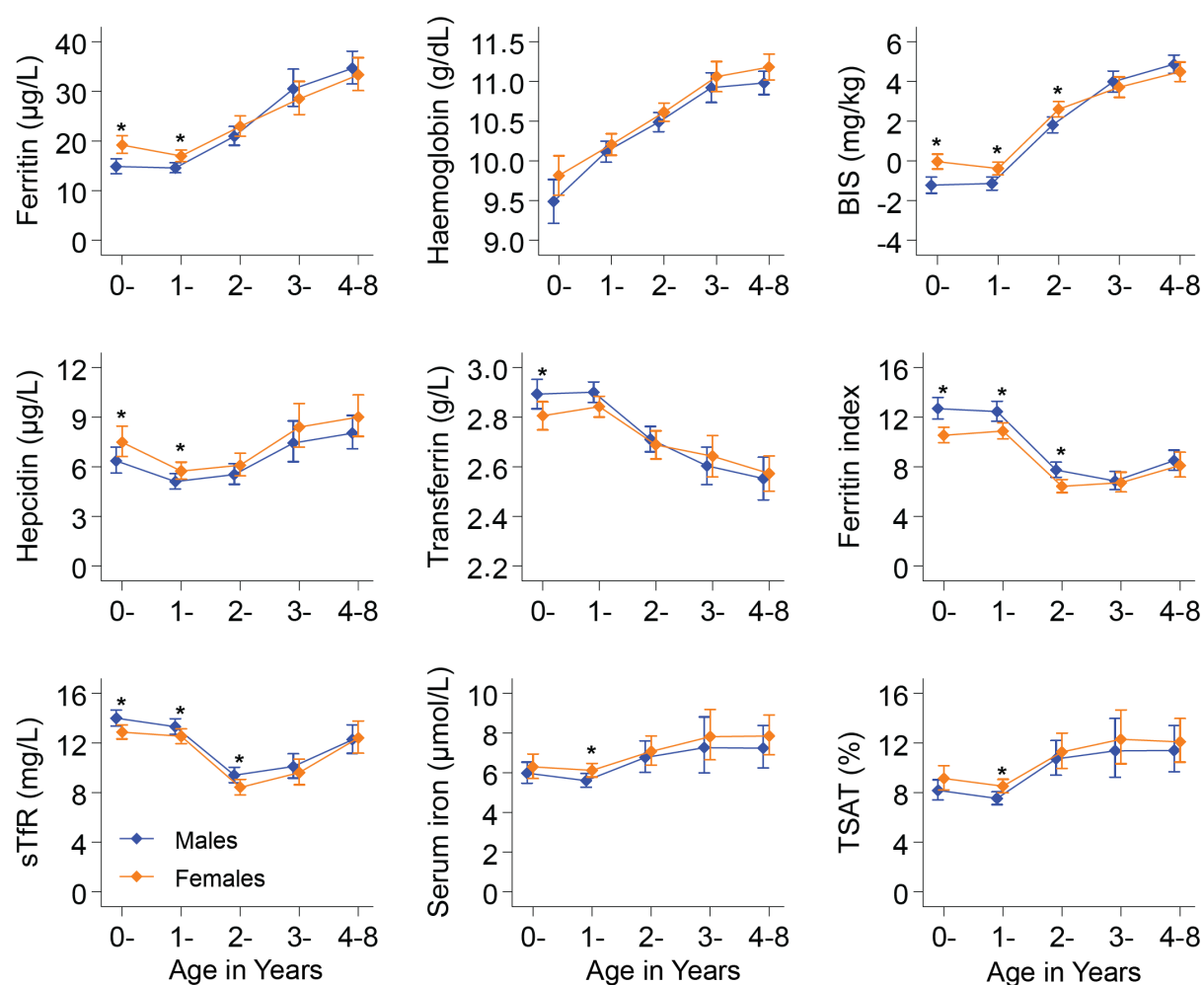


Figure 3.1. Geometric means for different iron biomarkers by age in years and sex.

Orange indicates females and blue males. Error bars indicate 95% confidence intervals. Star indicates a Student's *t*-test *p* value < 0.05 for mean differences between sexes. I calculated body iron stores (BIS) as proposed by Cook et al as $-(\log_{10}((sTfR \text{ in mg/L} \times 1000)/\text{ferritin in } \mu\text{g/L}) - 2.8229)/0.1207$ (Cook, Flowers and Skikne, 2003) and ferritin index as $sTfR \text{ in mg/L} / \log_{10}(\text{ferritin in } \mu\text{g/L})$ (Phiri, Calis, Siyasiya, et al., 2009). *sTfR*: soluble transferrin receptor; TSAT, transferrin saturation.

3.4.4 Associations between malnutrition and iron status

Malnutrition, as indicated by either stunting, underweight or wasting, was associated with altered markers of iron status in regression models adjusted for age, sex, and study site. Underweight and stunting were associated with lower concentrations of haemoglobin and BIS, and higher sTfR and ferritin index indicating increased iron deficiency (Figure 3.2). Additionally, underweight was associated with lower serum iron concentrations and stunting with higher ZPP concentrations (Figure 3.2). In a multivariable regression model, the effect of underweight on iron biomarkers was largely independent of inflammation (Figure 3.3).

3.4.5 Associations between inflammation and malaria and iron status

Inflammation and malaria were associated with increased concentrations of ferritin, hepcidin, sTfR, BIS and ZPP, and decreased haemoglobin and transferrin concentrations in regression models adjusted for age, sex and study site (Figure 3.2). Inflammation was associated with a small decrease in TSAT (log estimate -0.1; 95% CI: -0.2, 0.001; $P = 0.052$) while malaria was associated with a small increase in TSAT (log estimate = 0.1; 95% CI: 0.02, 0.2; $P = 0.015$, Figure 3.2). Malaria was independently associated with increased ferritin, sTfR and ZPP and decreased haemoglobin in multivariable regression models adjusted for inflammation, age, sex, and underweight. Inflammation was independently associated with almost all of the iron biomarkers, but not sTfR in models adjusted for malaria, age, sex and underweight. TSAT was not associated with inflammation or malaria in multivariable regression models (Figure 3.3).

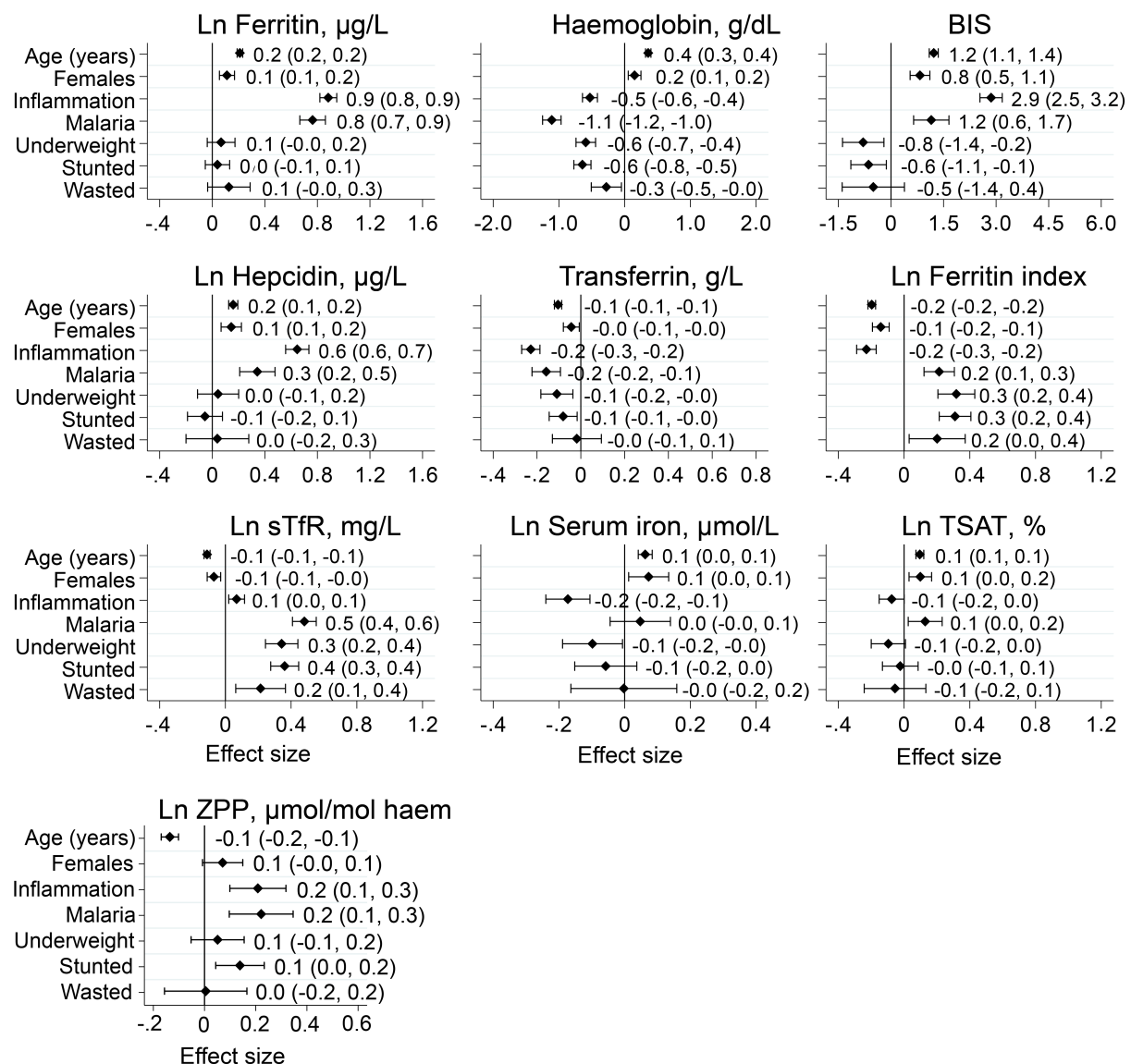


Figure 3.2. Predictors of iron biomarkers in regression models adjusted for age, sex and study site.

Effect size represents coefficient from linear regression model with the iron biomarker as the outcome variable. Iron biomarkers were ln-transformed except haemoglobin, transferrin and BIS. Independent variables were binary variables with exception of age, which was continuous. Error bars indicate 95% confidence intervals and values indicate effect size (95% CI). Inflammation was defined as C-reactive protein >5mg/L or α 1-antichymotrypsin >0.6 (in The Gambia). Malaria was defined as *P. falciparum* parasitaemia. Underweight was defined as weight for age z-score < -2, stunting as height-for-age z-score < -2, and wasting as weight-for-height z-score < -2. BIS, body iron stores; sTfR: soluble transferrin receptor; TSAT, transferrin saturation; ZPP, zinc protoporphyrin (measured in The Gambia only).

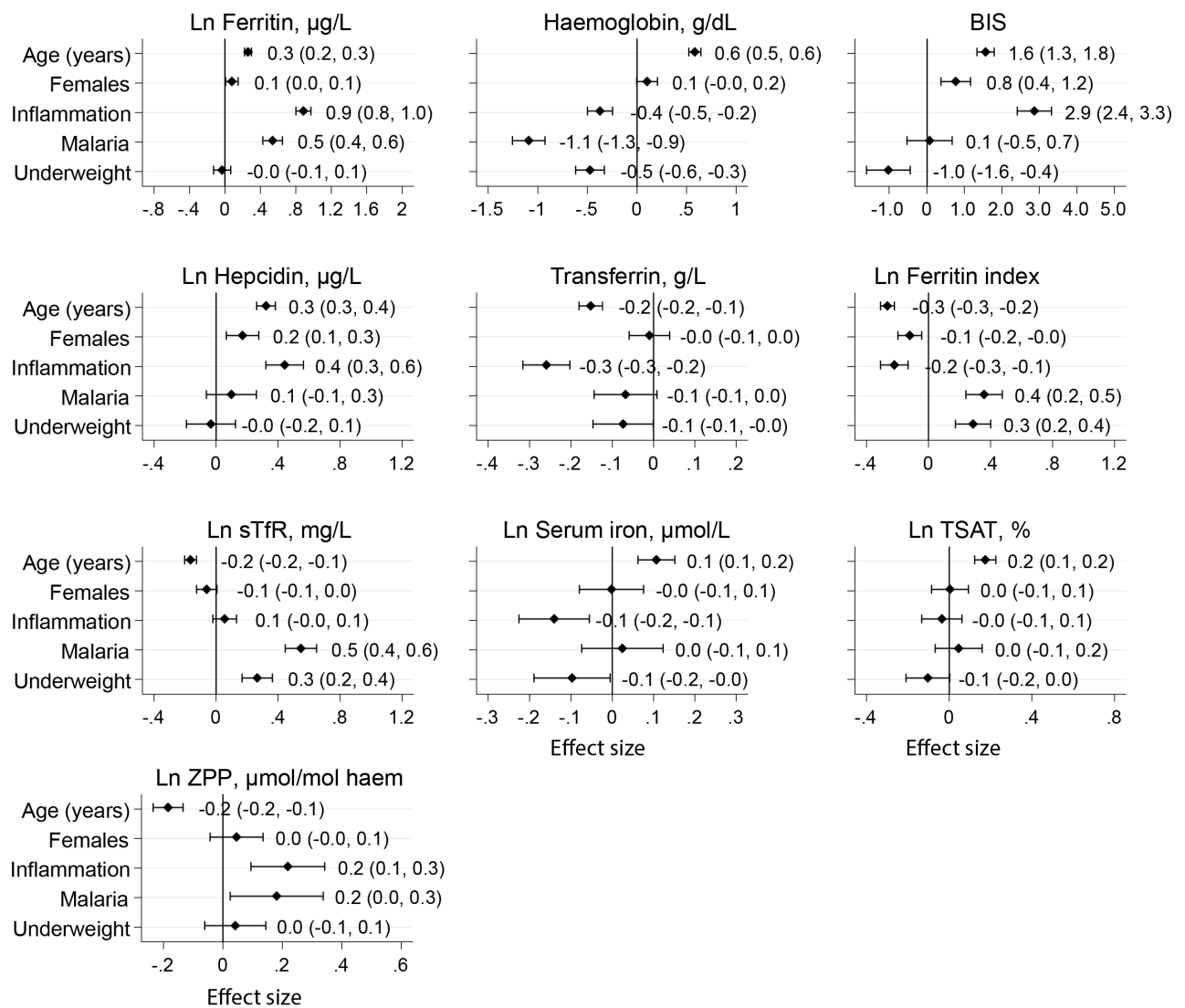
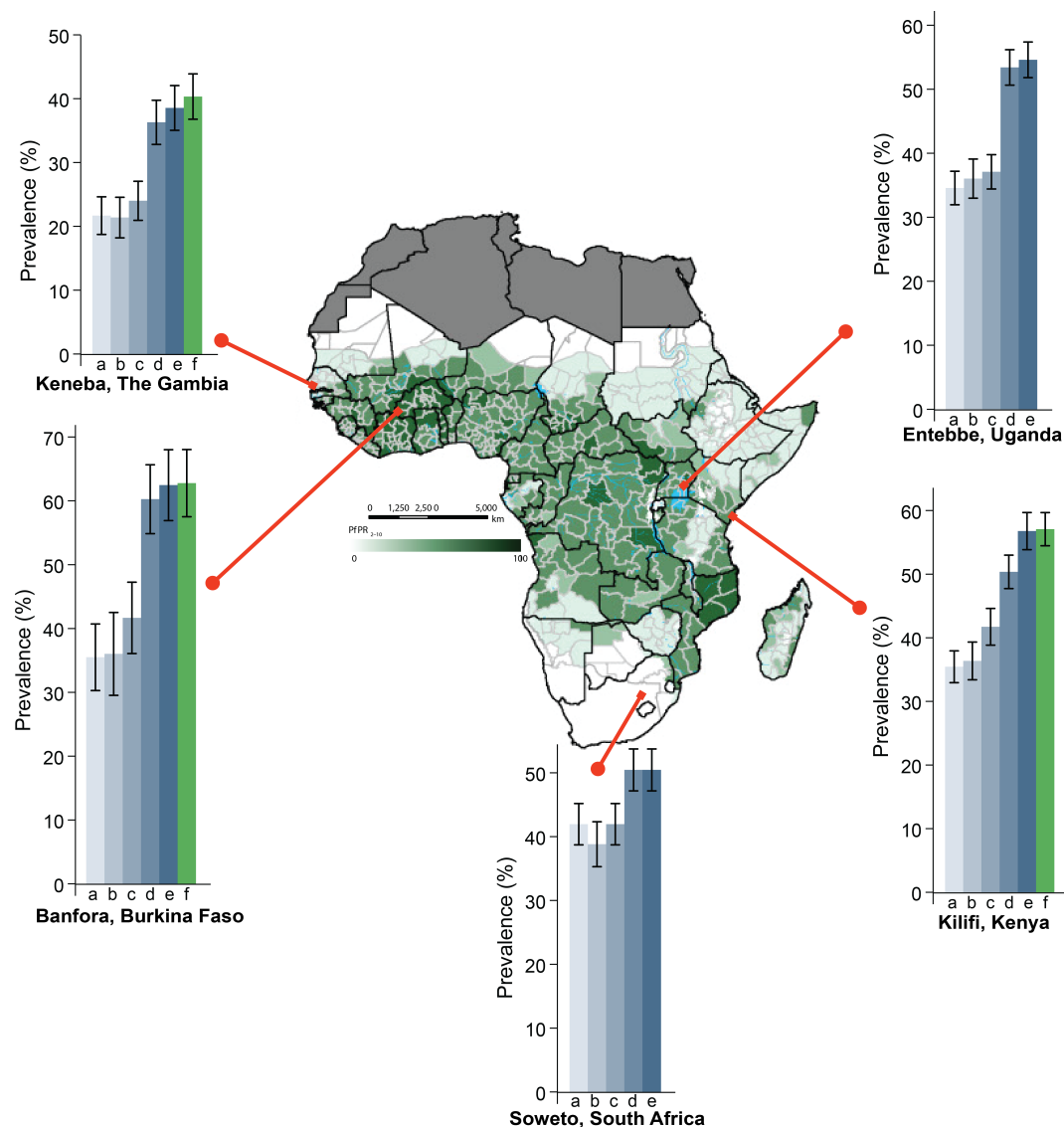


Figure 3.3. Predictors of iron biomarkers in multivariable regression models.

*Iron biomarkers were log transformed except haemoglobin, transferrin and BIS. Error bars indicate 95% confidence intervals and values indicate effect size (95% CI). Inflammation was defined as C-reactive protein > 5mg/L or α 1-antichymotrypsin > 0.6 (in The Gambia). Malaria was defined as *P. falciparum* parasitaemia. Underweight was defined as weight for age z-score < -2 and included to adjust for nutritional status. The South Africa cohort was excluded due to missing underweight. BIS, body iron stores; sTfR: soluble transferrin receptor; TSAT, transferrin saturation; ZPP, zinc protoporphyrin (measured in The Gambia only).*

3.4.6 Estimating the regression-corrected prevalence of iron deficiency

I then adjusted ferritin levels for inflammation and malaria parasitaemia using the regression-correction approach proposed by BRINDA (Namaste, Aaron, *et al.*, 2017; Namaste, Rohner, *et al.*, 2017). Figure 3.4 shows the prevalence of iron deficiency mapped on the African malaria map for the period 2010-2015 (Snow *et al.*, 2017). Excluding children with inflammation resulted in a similar prevalence of iron deficiency as WHO-defined iron deficiency. Adjustment of ferritin levels for inflammation alone substantially increased the prevalence of iron deficiency compared to adjustment for malaria alone, while adjustment for both malaria and inflammation led to a small further increase especially in Kenyan children who had the highest prevalence of malaria without inflammation (Figure 3.4). Further adjustments for age, sex and underweight did not change the prevalence of iron deficiency as shown in Figure 3.5. The pooled prevalence of iron deficiency after adjusting for both inflammation and malaria was 52.0% and the absolute increase in the prevalence of iron deficiency for each study site was: Burkina Faso, 27.0%; Kenya, 21.4%; Uganda, 20.0%; The Gambia, 16.8%; and South Africa, 8.5% (Figure 3.4). The gap between WHO-defined iron deficiency and regression-correction was highest in cohorts that had the highest prevalence of malaria and inflammation (Kenya and Burkina Faso) and lowest in malaria-free South Africa. The prevalence of iron deficiency defined by other iron biomarkers and by iron deficiency anaemia similarly increased following regression-correction for inflammation and malaria (Table 3.3). However, the prevalence of iron deficiency based on these other iron biomarkers varied widely underscoring a need for standardised assays and cut-offs to define iron deficiency.



| Study | Malaria only (%) | Inflammation only (%) | Malaria and inflammation (%) | WHO-defined ID (%) | Regression-corrected ID (%) | Absolute increase in ID (%) |
|--------------|------------------|-----------------------|------------------------------|--------------------|-----------------------------|-----------------------------|
| Burkina Faso | 8.9 | 22.4 | 11.5 | 35.5 | 62.5 | 27.0 |
| Kenya | 12.1 | 17.0 | 9.7 | 35.4 | 56.8 | 21.4 |
| Uganda | 3.0 | 19.4 | 4.0 | 34.6 | 54.6 | 20.0 |
| The Gambia | 8.0 | 11.6 | 3.2 | 21.7 | 38.5 | 16.8 |
| South Africa | 0.0 | 17.6 | 0.0 | 42.0 | 50.5 | 8.5 |
| Pooled | 6.0 | 17.3 | 5.1 | 34.2 | 52.0 | 17.8 |

Figure 3.4. Prevalence of estimated iron deficiency across the study sites.

The map shows the predicted posterior predictions of age-standardised *P. falciparum* prevalence (PfPR₂₋₁₀) as previously published by (Snow et al., 2017). Map was reproduced with permission. Graph letter 'a' indicates prevalence of iron deficiency using WHO definition, 'b' excluding children with inflammation, 'c' adjusting for malaria only, 'd' adjusting for inflammation only, 'e' adjusting for both malaria and inflammation, and 'f' using transferrin saturation cut-off of <11%. Values indicate prevalence. Absolute increase in iron deficiency was calculated as the difference between regression-corrected (for both malaria and inflammation) prevalence and WHO-defined prevalence. Error bars indicate 95% confidence intervals.

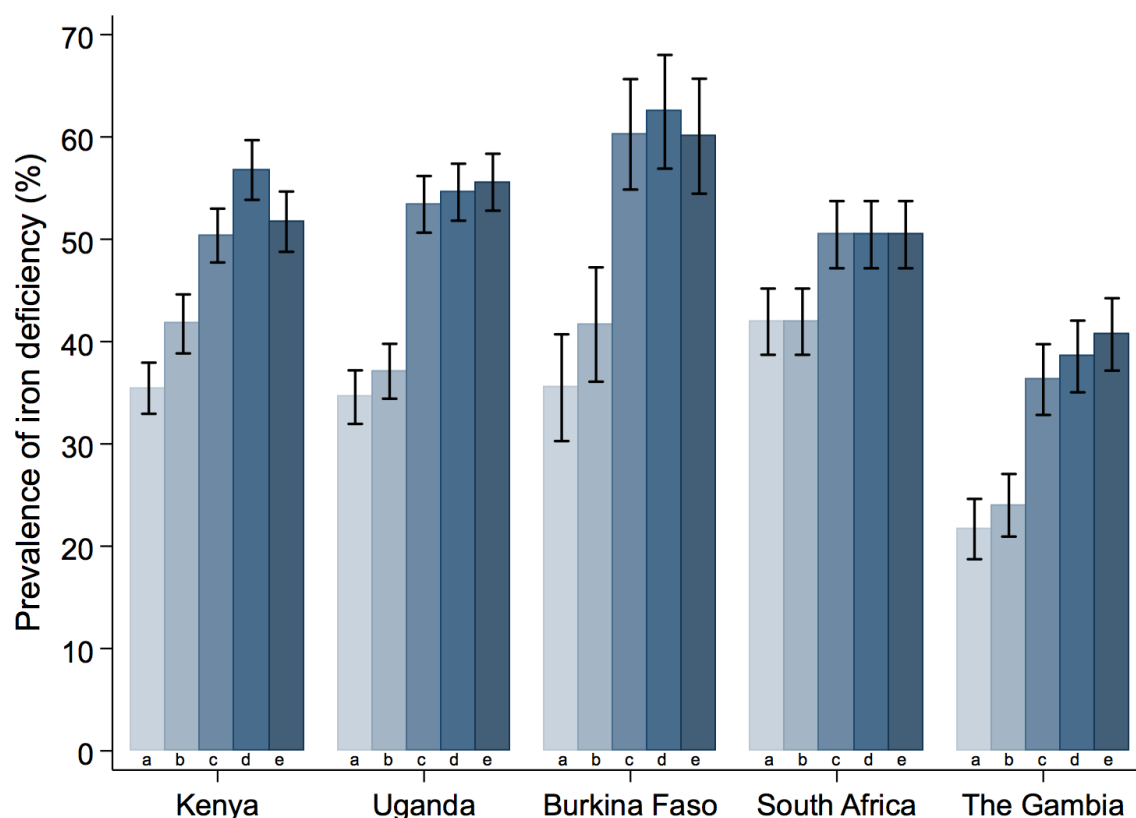


Figure 3.5. Comparison of prevalence of iron deficiency after additional adjustments for age, sex and nutritional status.

Graph letter 'a' indicates prevalence using WHO definition, 'b' adjusting for malaria only, 'c' adjusting for inflammation only, 'd' adjusting for both malaria and inflammation, and 'e' adjusting for malaria, inflammation, age, sex and underweight. Error bars indicate 95% confidence intervals. No malaria in South Africa. Anthropometry data were unavailable for South Africa.

Table 3.3. Regression-corrected and uncorrected prevalence of iron deficiency defined by additional iron biomarkers by study site

| Definition | Kenya | | | Uganda | | | Burkina Faso | | | South Africa | | | The Gambia | | | Pooled | | |
|--|-------|------|------------|--------|------|------------|--------------|------|------------|--------------|------|------------|------------|------|------------|--------|------|------------|
| | n | % | 95% CI | n | % | 95% CI | n | % | 95% CI | n | % | 95% CI | n | % | 95% CI | n | % | 95% CI |
| <u>ID - sTfR >8.3mg/L</u> | | | | | | | | | | | | | | | | | | |
| Uncorrected | 1467 | 97.9 | 97.2, 98.6 | 1343 | 36.9 | 34.3, 39.5 | 342 | 94.2 | 91.7, 96.7 | 893 | 80.4 | 77.8, 83.0 | 661 | 1.7 | 0.7, 2.6 | 4706 | 63.4 | 62.0, 64.8 |
| Inflammation/malaria corrected | 1140 | 96.8 | 95.8, 97.9 | 1292 | 33.1 | 30.6, 35.7 | 302 | 94.4 | 91.8, 97.0 | 893 | 80.9 | 78.4, 83.5 | 659 | 1.5 | 0.6, 2.5 | 4286 | 59.5 | 58.0, 61.0 |
| <u>Hepcidin <3.2µg/L</u> | | | | | | | | | | | | | | | | | | |
| Uncorrected | 1373 | 31.7 | 29.2, 34.1 | 1333 | 26.0 | 23.6, 28.3 | 309 | 34.6 | 29.3, 40.0 | 878 | 22.9 | 20.1, 25.7 | 709 | 27.8 | 24.5, 31.1 | 4602 | 27.9 | 26.6, 29.2 |
| Inflammation/malaria corrected | 1075 | 51.3 | 48.3, 54.2 | 1282 | 35.2 | 32.6, 37.8 | 276 | 50.0 | 44.1, 55.9 | 878 | 31.4 | 28.4, 34.5 | 700 | 33.6 | 30.1, 37.1 | 4211 | 39.2 | 37.7, 40.7 |
| <u>Depleted BIS (<0mg/kg)¹</u> | | | | | | | | | | | | | | | | | | |
| Uncorrected | 1393 | 54.1 | 51.4, 56.7 | 1241 | 28.1 | 25.6, 30.6 | 322 | 55.9 | 50.4, 61.4 | 893 | 48.5 | 45.2, 51.8 | 660 | 7.3 | 5.3, 9.3 | 4509 | 39.1 | 37.7, 40.5 |
| Inflammation/malaria corrected | 1098 | 77.9 | 75.4, 80.3 | 1205 | 45.2 | 42.4, 48.0 | 291 | 82.5 | 78.1, 86.9 | 893 | 64.2 | 61.0, 67.3 | 658 | 12.8 | 10.2, 15.3 | 4145 | 55.4 | 53.9, 56.9 |
| <u>Ferritin index >5.6²</u> | | | | | | | | | | | | | | | | | | |
| Uncorrected | 1392 | 96.4 | 95.4, 97.4 | 1241 | 48.9 | 46.1, 51.7 | 322 | 91.6 | 88.6, 94.7 | 893 | 85.1 | 82.8, 87.4 | 660 | 88.9 | 86.5, 91.3 | 4508 | 79.7 | 78.5, 80.8 |
| Inflammation/malaria corrected | 1098 | 96.5 | 95.4, 97.6 | 1205 | 57.7 | 54.9, 60.5 | 291 | 95.2 | 92.7, 97.7 | 893 | 90.3 | 88.3, 92.2 | 658 | 91.9 | 89.9, 94.0 | 4145 | 83.1 | 81.9, 84.2 |
| <u>Iron deficiency anaemia³</u> | | | | | | | | | | | | | | | | | | |
| Uncorrected | 833 | 24.8 | 21.9, 27.8 | 1209 | 21.1 | 18.8, 23.4 | 309 | 31.1 | 25.9, 36.3 | n/a | n/a | n/a | 745 | 16.5 | 13.8, 19.2 | 3096 | 22.0 | 20.5, 23.5 |
| Inflammation/malaria corrected | 731 | 41.3 | 37.7, 44.9 | 1177 | 30.8 | 28.1, 33.4 | 290 | 55.5 | 49.8, 61.3 | n/a | n/a | n/a | 735 | 27.6 | 24.4, 30.9 | 2933 | 35.0 | 33.3, 36.8 |

BIS, body iron stores; ZPP, zinc protoporphyrin; n/a, not available.

Regression-correction for inflammation and malaria were made on individual iron biomarkers and then used to define iron deficiency.

¹Body iron stores were calculated using the ratio of soluble transferrin receptors (sTfR) and ferritin concentrations (Cook, Flowers and Skikne, 2003).

²Ferritin index defined as sTfR/log10 ferritin (Phiri, Calis, Siyasiya, *et al.*, 2009).

³Iron deficiency anaemia was defined as iron deficiency and anaemia.

3.4.7 Misclassification of iron-deficient children increases with age, inflammation and malaria

The gap between the prevalence of WHO-defined iron deficiency and regression-corrected iron deficiency increased with age, increasing prevalence of malaria parasitaemia (Figure 3.6) and with increasing CRP levels (Figure 3.7A). Prevalence of regression-corrected iron deficiency remained relatively constant over the spectrum of CRP levels, while prevalence of WHO-defined iron deficiency decreased linearly above the third decile of CRP (0.4 mg/L), before adjustment for inflammation (CRP > 5 mg/L) (Figure 3.7A).

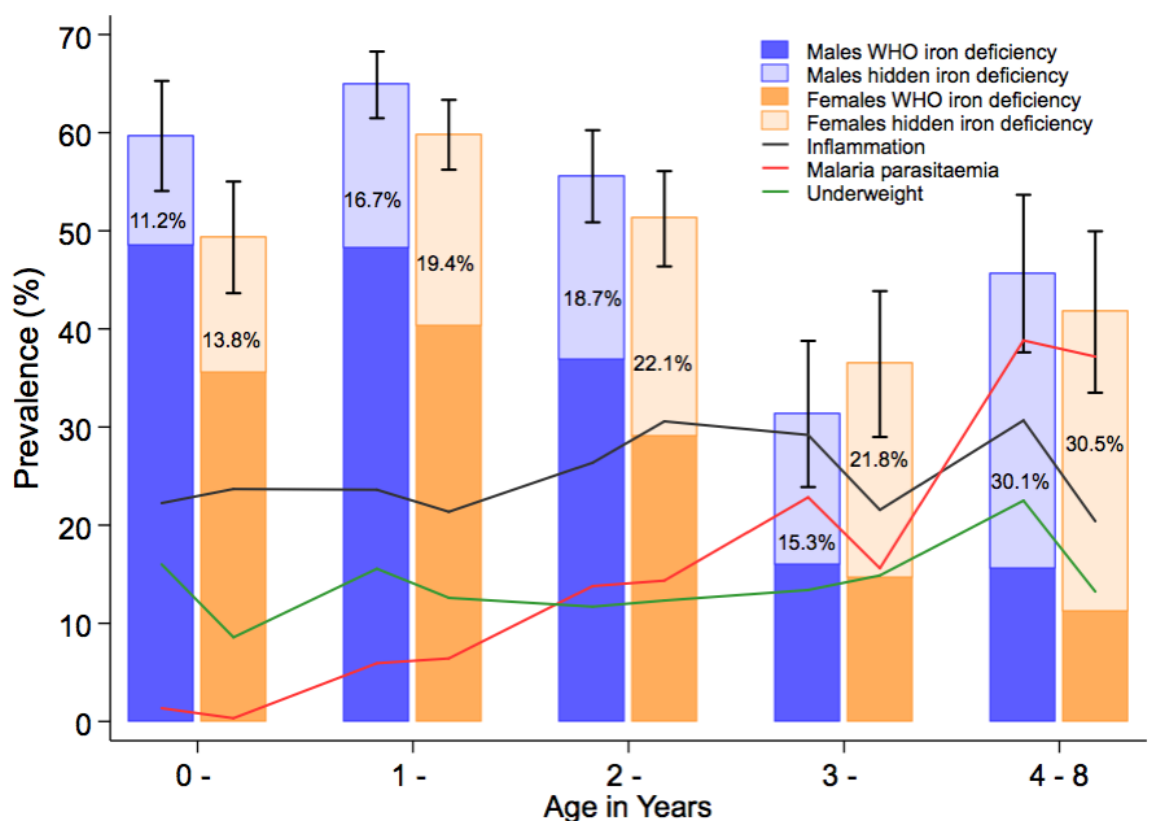


Figure 3.6. The hidden burden of iron deficiency by sex and age.

Error bars indicate 95% confidence intervals for adjusted WHO definition. Darker colours indicate the WHO definition of iron deficiency while lighter colours show the hidden burden after adjustment for malaria and inflammation. The values in the bars indicate the percentage of children with iron deficiency unaccounted for by the WHO definition (a 'hidden burden'). Line plots indicate how the prevalence of inflammation (black), malaria (red) and underweight (green) changed with age.

Malaria may also contribute to underestimation of prevalence of iron deficiency. Malaria-endemic countries had a higher percentage of children misclassified as iron-replete (27.0% in Burkina Faso compared to 8.5% in South Africa; Fig. 3). Children with malaria also had higher ferritin concentrations at every decile of CRP, compared to those without malaria (Figure 3.7B). Moreover, the gap between WHO-defined and regression-corrected prevalence of iron deficiency was larger in children with malaria parasitaemia compared to those without, regardless of the presence of inflammation (Figure 3.8).

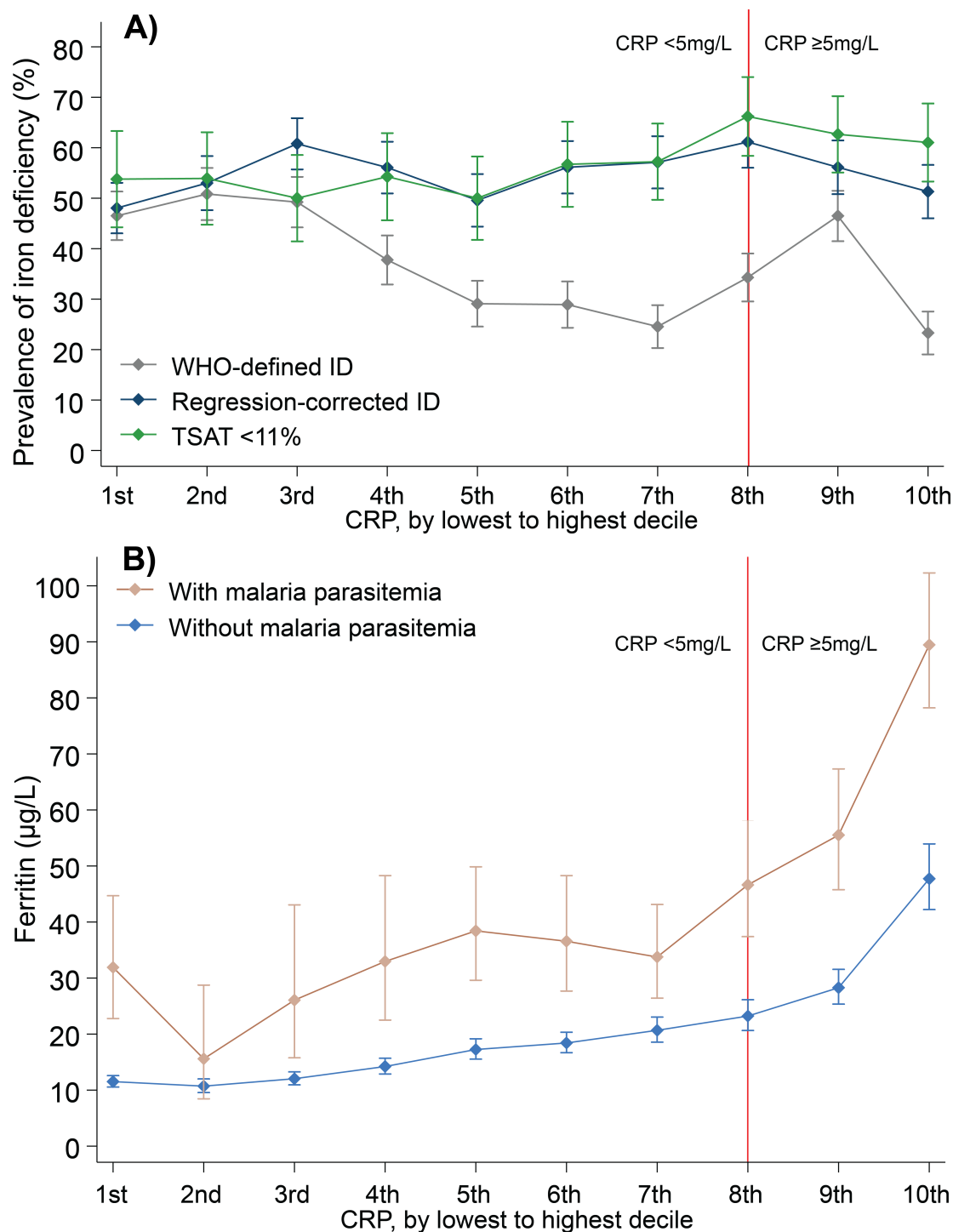


Figure 3.7. Relationship between the estimated prevalence of iron deficiency and inflammation / malaria.

A) shows how the prevalence of various estimates of iron deficiency varied by deciles of C-reactive protein (CRP). **B)** shows that ferritin levels were higher in children with malaria parasitaemia compared to those without parasitaemia at every CRP decile. Error bars indicate 95% confidence intervals. Low ferritin was defined as ferritin <12 µg/L in children <5 years or <15 µg/L in children ≥5 years. WHO adjustment involved using a higher cut-off (30 µg/L) of ferritin for children with CRP ≥5mg/L. Regression adjustment indicates that the effect of inflammation and malaria were adjusted for using regression modelling. TSAT, transferrin saturation.

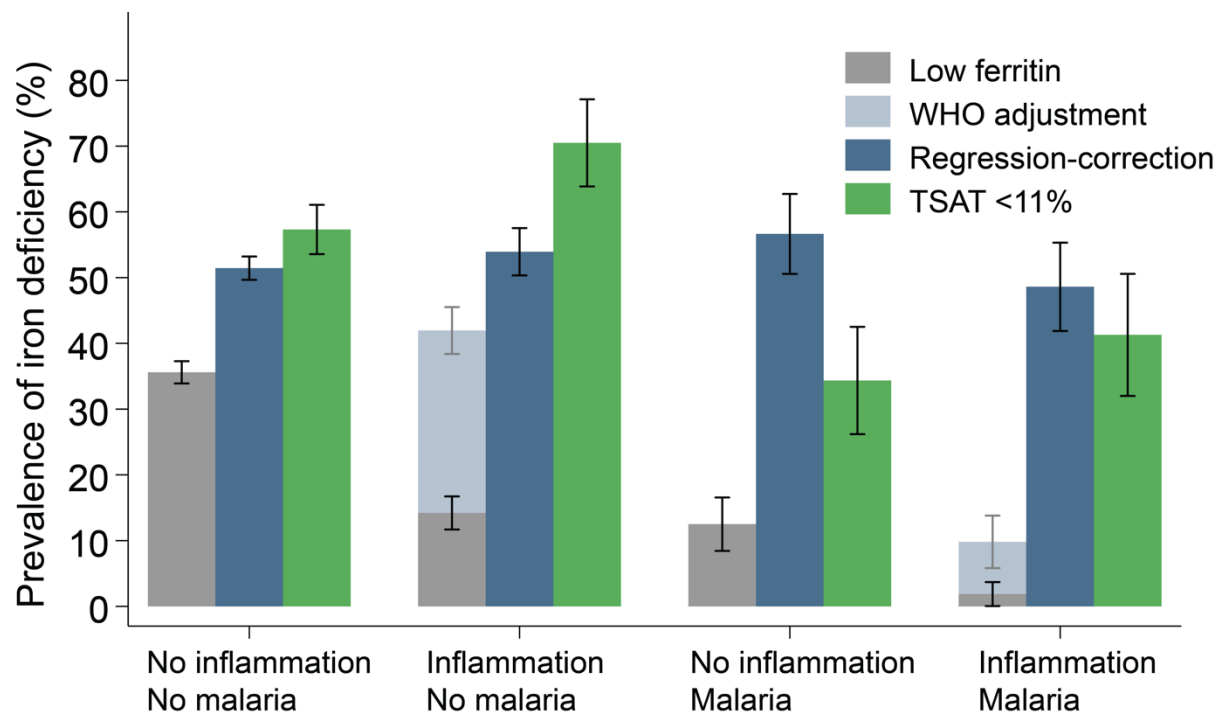


Figure 3.8. Relationship between the estimated prevalence of iron deficiency and inflammation / malaria.

*The graph shows the prevalence of iron deficiency in children with and without inflammation and / or malaria. Error bars indicate 95% confidence intervals. Inflammation was defined as C-reactive protein > 5mg/L or α 1-antichymotrypsin > 0.6g/dL (in The Gambia). Low ferritin was defined as ferritin <12 μ g/L in children <5 years or <15 μ g/L in children \geq 5 years. WHO adjustment involved using a higher cut-off (30 μ g/L) of ferritin for children with inflammation according to WHO-defined iron deficiency. Malaria was defined as *P. falciparum* parasitaemia. Regression-correction uses ferritin levels corrected for the effects of inflammation and malaria in defining ID. TSAT, transferrin saturation.*

3.4.8 Diagnostic utility of iron biomarkers in predicting regression-corrected iron deficiency

Finally, I used the regression-corrected iron deficiency (corrected for the effects of inflammation and malaria on ferritin levels) as a gold standard to test the diagnostic utility of various markers of iron status (Figure 3.9). TSAT outperformed other markers of iron status. For TSAT, I observed an area under curve (AUC) of 0.77 and an optimal cut-off of 10.56, similar to the overall cut-off (11.11) that was obtained from a meta-analysis of cohort-specific optimal cut-offs (Figure 3.10). I then applied a rounded cut-off of TSAT <11% and obtained similar prevalence of iron deficiency as that obtained using the regression-corrected definition of iron deficiency (green bar in Figure 3.4 above). TSAT <11% also performed well across the spectrum of CRP levels with only a modest increase in prevalence of iron deficiency compared to regression-corrected iron deficiency during inflammation (shown Figure 3.7A above). Other iron biomarkers did not perform as well in predicting regression-corrected iron deficiency. Haemoglobin concentrations had an AUC of 0.61 and an optimal cut-off of 11.25 g/dL with a sensitivity of 75%, but low specificity of 42%, whilst sTfR concentrations had low sensitivity (43%) in predicting iron deficiency corrected for inflammation and malaria (Figure 3.9).

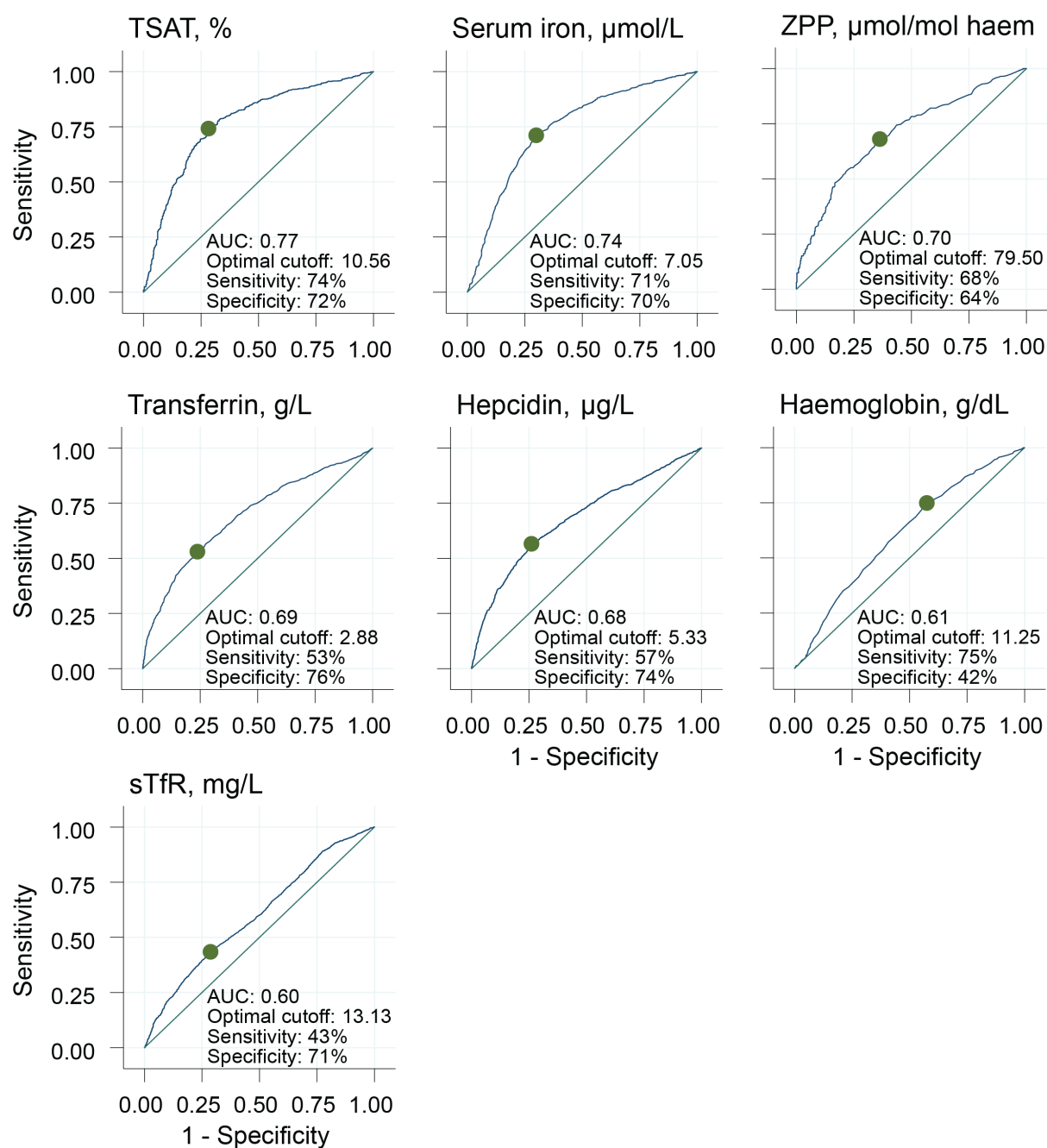


Figure 3.9. Receiver operating characteristic curves of the utility of iron markers in estimating adjusted prevalence of iron deficiency.

The ‘gold standard’ was defined using the WHO definition adjusted for malaria and inflammation using regression modeling. Green points indicate Youden’s optimal cutoffs for each marker. Sensitivity and specificity are for the optimal cutoff. TSAT, Transferrin saturation; MCV, mean corpuscular volume; ZPP, zinc protoporphyrin; sTfR, soluble transferrin receptor; AUC, area under curve.

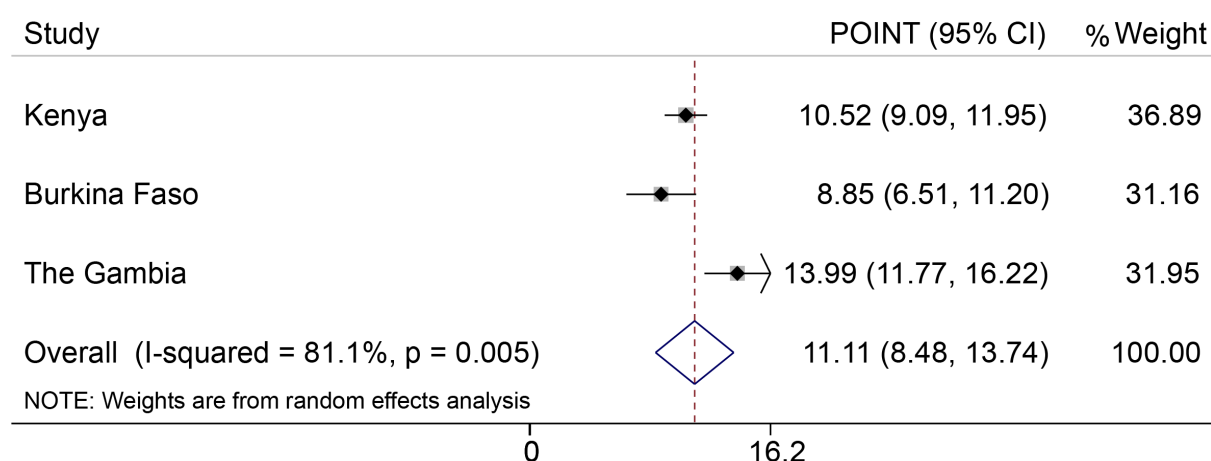


Figure 3.10. Meta-analysis of optimal cutoffs of transferrin saturation in predicting regression adjusted iron deficiency.

3.5 Discussion

In this chapter, I have described determinants of iron status and estimated the burden of iron deficiency in approximately 4,800 children living across the African continent using a wide range of iron biomarkers. Iron deficiency increased from birth to around one year, and then decreased with increasing age among infants. Iron deficiency was higher in males compared to females up to three years of age. Stunting and underweight were associated with increased iron deficiency and erythropoietic drive. Inflammation and malaria parasitaemia were associated with independent and substantial increases in ferritin concentrations and were associated with other biomarkers of iron status. The WHO definition of iron deficiency underestimated the burden of iron deficiency in African children compared to regression-correction, which predicts ferritin levels in the absence of inflammation and malaria. Of the other iron markers that I tested, TSAT was least influenced by inflammation, malaria, or malnutrition and had the best diagnostic properties when compared against the gold standard of regression-correction.

Age and sex predicted measures of iron status especially up to three years of age. Iron stores decreased rapidly during infancy and then increased during childhood, supporting the argument that newborns have higher iron stores that are prenatally accumulated but decrease with the

demand for iron during rapid growth (Wu, Lesperance and Bernstein, 2002; Ziegler, Nelson and Jeter, 2014; Joo *et al.*, 2016). The prevalence of iron deficiency was highest at around one year of age, probably because of rapid child growth at a time when iron supply from breast milk and complementary foods is low (Wu, Lesperance and Bernstein, 2002; Ziegler, Nelson and Jeter, 2014). Similarly, a recent study in Gambian children showed declining iron status of infants monitored from birth up to one year (Armitage *et al.*, 2019). Moreover, weight gain was strongly associated with decrease in hepcidin levels suggesting that rapid growth is associated with iron depletion (Armitage *et al.*, 2019). Iron status improved during childhood, possibly due to diversifying diets and growth rate slowing after infancy (Wu, Lesperance and Bernstein, 2002). In agreement with previous studies (Tamura *et al.*, 1999; Domellof *et al.*, 2002; Jaeggi *et al.*, 2013; Ziegler, Nelson and Jeter, 2014; Armitage *et al.*, 2019), female infants were less iron deficient than males. This sex difference might be explained by hormonal hepcidin regulation since testosterone is associated with suppression of hepcidin (Bachman *et al.*, 2010) and lower ferritin levels (Chao *et al.*, 2015). These results highlight important age and sex differences in iron status among infants.

Poor nutrition was associated with iron deficiency. Children with stunting or underweight had lower body iron stores, serum iron and haemoglobin concentrations, and higher sTfR, ferritin index and ZPP, in keeping with increased iron deficiency. I observed similar associations even after controlling for inflammation. These findings suggest that malnourished children are more likely to experience iron deficient erythropoiesis (Labbé and Dewanji, 2004). I also found that malnourished children were more likely to have malaria parasitaemia, which may in turn lead to increased erythropoiesis (Menendez *et al.*, 2001). Furthermore, malaria-associated erythropoiesis has been shown to be more common in stunted than in non-stunted children (Verhoef, West, Veenemans, *et al.*, 2002). Malnutrition was also associated with decreased

haemoglobin concentrations, which may in turn have led to expanded erythropoiesis (Foote *et al.*, 2013). Thus, these findings suggest that improving the nutritional status of children may also help to address iron deficiency and anaemia.

Inflammation and malaria parasitaemia were substantially associated with measures of iron status in agreement with previous studies (Aguilar *et al.*, 2012; Righetti *et al.*, 2013; Namaste, Rohner, *et al.*, 2017). Importantly, I found that both inflammation and malaria parasitaemia were independently associated with increased levels of ferritin, thus, potentially leading to children being misclassified as iron replete. I therefore redefined iron deficiency by correcting ferritin levels for the effects of inflammation and malaria parasitaemia using regression-correction, as previously proposed by BRINDA (Namaste, Rohner, *et al.*, 2017). The strength of this approach is that it accounts for continuous measures of inflammation as opposed to the arbitrary cut-off points used by WHO (World Health Organization, 2011b). I found that the WHO definition substantially underestimated the prevalence of iron deficiency among children living in sub-Saharan Africa compared to the inflammation and malaria-corrected definition of iron deficiency. The underestimate was particularly higher after adjusting for inflammation only and the independent effect of inflammation on ferritin levels was also greater than that of malaria parasitaemia. Adjustment for malaria parasitaemia in addition to inflammation resulted in a slight increase in the prevalence of iron deficiency. Using a similar approach, Namaste *et al.* observed similar absolute percentage increases (of up to 27%) in children misclassified as iron-replete using WHO-defined iron deficiency compared to regression-corrected iron deficiency (Namaste, Rohner, *et al.*, 2017).

The underestimate of prevalence of iron deficiency by WHO was largest in cohorts that had a high burden of malaria parasitaemia. For example, 27.0% of Burkinabe children and 21.4% of

Kenyans were misclassified as iron replete compared to 8.5% of South African children. The underestimate of the burden of iron deficiency increased with age in line with increasing prevalence of parasitaemia. Moreover, ferritin levels were higher among children with malaria parasitaemia at every decile of CRP. Malaria parasitaemia also increased ferritin levels independently of inflammation in multivariable analyses. In agreement, a study in Burkinabe children found that adjustment for asymptomatic malaria, in addition to inflammation, led to an increase in the prevalence of iron deficiency by 15.9 percentage points (Wessells and Hess, 2014). These findings suggest that both inflammation and malaria parasitaemia should be accounted for in population estimates of prevalence of iron deficiency in African children.

Finally, I evaluated the diagnostic utility of uncorrected iron biomarkers in predicting iron deficiency regression-corrected for inflammation and malaria. I found that TSAT <11% best predicted regression-corrected iron deficiency suggesting its potential usefulness in estimating the prevalence of iron deficiency in African children. TSAT can be calculated from measured serum iron and either transferrin or UIBC, all of which have standard assays that are easy and inexpensive to perform. TSAT <11% performed well over a range of CRP concentrations, in children with both malaria parasitaemia and inflammation, and across populations. In support of my findings, Aguilar *et al* showed that TSAT had a high sensitivity (81%) in predicting bone marrow iron deficiency in 180 anemic (haemoglobin <11g/dL) children in Mozambique although specificity was low (40%) and an optimal cut-off was not derived (Aguilar *et al.*, 2012). In contrast, another study in Malawian children showed limited value of TSAT in diagnosing bone marrow iron stores although children in this study were severely anaemic (haemoglobin <5g/dL) (Phiri, Calis, Siyasiya, *et al.*, 2009). Other iron markers did not perform as well as TSAT in predicting regression-corrected iron deficiency. Notably, sTfR, which is

normally proposed as an alternative indicator of iron deficiency in presence of infections, had very low sensitivity (43%).

Anaemia may also be a poor indicator of the burden of iron deficiency. WHO recommends iron supplementation in populations where the prevalence of anaemia is $\geq 40\%$ (World Health Organization, 2016). However I found that haemoglobin concentrations had very low specificity (42%) for predicting regression-corrected iron deficiency probably because of the multifactorial etiology of anaemia in African children (Foote *et al.*, 2013). Based on this guideline, all children in my study populations would have received iron, although approximately half were iron-replete. Therefore, TSAT may be a better marker than haemoglobin for determining the prevalence of iron deficiency in African children although a more sensitive and specific marker is needed.

These analyses had a number of important limitations. The cross-sectional nature of the data limited analyses of longitudinal effects of inflammation, malaria and nutritional status on iron status. Furthermore, I did not have data on α -1-acid glycoprotein (AGP), which has been shown to be a better marker for adjusting for inflammation in regression-correction analyses (Namaste, Rohner, *et al.*, 2017). Nevertheless, unlike AGP, CRP is more widely measured and international reference standards are available. I used iron deficiency regression-corrected for inflammation and malaria as the gold standard although this method is yet to be validated, for example by either comparing prevalence estimates of regression-correction before, during and after infections or with bone marrow iron deficiency. I used a ferritin-based definition of iron deficiency since other iron biomarkers have less standardised assays and less well-established cut-offs for iron deficiency. Another limitation of my analyses was that TSAT, the best performing marker in predicting regression-corrected iron deficiency was not available for

Ugandan and South African children. Moreover, although TSAT outperformed all other iron markers it had an AUC of only 0.77 which may be lower than is expected of a clinical diagnostic test.

3.6 Conclusion

Using data for more than 4,800 children in five countries across Africa, I determined the drivers of iron status and estimated the burden of iron deficiency. I also explored a wide range of iron biomarkers to more accurately estimate prevalence of iron deficiency in countries with a high burden of childhood infections including malaria. There has been a long-standing concern regarding the challenge of using iron biomarkers to accurately estimate prevalence of iron deficiency in African populations (Suchdev *et al.*, 2017). In this study, I found that after accounting for the effects of inflammation and malaria parasitaemia on ferritin levels, the prevalence of iron deficiency was substantially higher in African children than can be estimated using the WHO definition. Of the measured iron biomarkers, TSAT was the best predictor of iron deficiency regression-corrected for inflammation and malaria parasitaemia. Therefore, TSAT may be useful in estimating prevalence of iron deficiency to guide planning and implementation of nutritional iron interventions, since the regression-correction approach would not be practical for programmatic screening of children in routine clinical care. However, further research is required to validate the regression-correction approach against a gold standard for more accurate interpretation of existing iron biomarkers and / or to identify easy to use, more sensitive and specific markers of iron status that are not influenced by infection.

CHAPTER FOUR: INVESTIGATING WHETHER MALARIA CAUSES IRON DEFICIENCY IN AFRICAN CHILDREN

The material in this chapter forms the basis of a submitted manuscript entitled “*Malaria is a cause of iron deficiency in African children*”, in which I am the first author.

4.1 Abstract

Introduction: Previous observational studies suggest that malaria may cause iron deficiency in African children. The link between malaria and iron deficiency may be explained by the iron hormone hepcidin which is upregulated during malaria infection and blocks iron absorption. I tested the hypothesis that malaria causes iron deficiency in 7453 African children using sickle cell trait (HbAS), a common genetic variant conferring protection against clinical malaria, as an instrumental variable in Mendelian randomisation analyses.

Methods: I used data on measures of iron status, inflammation and sickle cell genotype from 10 African community-based cohorts of children aged 0-8 years from Malawi, Ghana, Burkina Faso, Democratic Republic of Congo (DRC), Kenya (Kilifi and Western Kenya), Tanzania, The Gambia, Cameroon and Uganda. I fitted cohort-specific regression models to determine the effect of HbAS on iron status then conducted meta-analyses and Mendelian randomisation analyses. I tested for pleiotropy (i.e. whether HbAS influences iron deficiency through malaria independent pathways) using a malaria-free cohort of 3207 African-Americans from the Jackson Heart study.

Results: In malaria-exposed children, HbAS was associated with a 30% reduction in iron deficiency (OR = 0.70; 95% CI 0.58, 0.82; $P < 0.001$). HbAS was not associated with measured confounders or with iron deficiency in 3207 non-malaria exposed African-Americans. In Mendelian randomisation analysis, genetically influenced malaria risk was associated with a 2.52 (95% CI 1.60, 3.98; $P < 0.001$) odds of iron deficiency per unit increase in log incidence

rate of malaria. This suggests that an intervention that halves the risk of malaria episodes would reduce iron deficiency by 47% in African children.

Conclusion: These findings suggest that malaria might be causing iron deficiency and support implementation of malaria control and treatment programs to combat iron deficiency in African children.

4.2 Introduction

Malaria and iron deficiency tend to co-exist in the same geographic and demographic groups. Although malaria causes anaemia by the destruction of infected and uninfected RBCs, the iron released from the destroyed RBCs is not lost from the body as it is recycled by macrophages. The iron hormone, hepcidin, may link malaria and iron deficiency since hepcidin is up-regulated during clinical and asymptomatic malaria and blocks iron absorption and recycling (discussed in Chapter One). I therefore hypothesised that malaria may be causing iron deficiency in African children. To determine whether malaria causes iron deficiency, a number of epidemiological approaches can be applied although with some limitations (discussed in Chapter One Section 1.3.2.1).

In this chapter, I test the hypothesis that malaria may be causing iron deficiency in African children by applying a Mendelian randomisation approach (discussed in Chapter One Section 1.4). I utilised sickle cell trait (HbAS), a genetic variant conferring protection against malaria (Taylor, Parobek and Fairhurst, 2012), to proxy malaria exposure. I analysed data for 7453 African children aged 0-8 years from ten community-based cohorts across Africa. To test whether HbAS might influence risk of iron deficiency through a mechanism that is independent of its effect on malaria (pleiotropy), I repeated the analyses in 3207 non-malaria exposed African Americans from the Jackson Heart Study (JHS) as a no-relevance point sensitivity

analysis (Chen *et al.*, 2008). I also assessed the effect of other malaria-protective polymorphisms including glucose-6-phosphate dehydrogenase (G6PD) deficiency and α -thalassaemia.

4.3 Methods

4.3.1 Study populations

In this chapter, I used data for iron and inflammatory markers and sickle cell / G6PD / α -thalassaemia genotypes data from 10 cohorts of malaria-exposed children from the *iron_vaccgene* studies (Kilifi, Kenya; Banfora, Burkina Faso; Entebbe, Uganda; and Soweto, South Africa), West Kiang, The Gambia, and from six additional studies that are described below. I also included data from a hospital-based study of severe malaria in Kilifi and from a malaria-unexposed population of African-Americans from the Jackson Heart Study (JHS).

Malawi: I used data from the 2015-2016 Malawi Micronutrient Survey (MMS) that was conducted as part of the Malawi Demographic and Health Survey with an aim of assessing the prevalence of anaemia, micronutrient deficiencies, including iron deficiency, infections and haemoglobinopathies (McGann *et al.*, 2018). The MMS included all children aged 6-59 months from randomly selected clusters and households (McGann *et al.*, 2018). Venipuncture whole blood collected in EDTA tubes was used to test for malaria using rapid diagnostic test (RDT) (SD BIOLINE Malaria *P. falciparum* [HRP2], Alere, Inc., Waltham, MA) and to measure haemoglobin concentrations using the HemoCue 301 (Hemocue America, Brea, CA). Serum ferritin, CRP, and α 1-acid glycoprotein (AGP) were measured using sandwich enzyme-linked immunoassay (ELISA, VitMin Laboratory, Germany) (Erhardt *et al.*, 2004). Genotyping of sickle cell, α -thalassaemia and G6PD deficiency was performed using TaqMan Polymerase Chain Reaction (PCR) as detailed in (McGann *et al.*, 2018).

Ghana: This study was part of the 2017 Ghana Micronutrient Survey. Details of the study design and ethical approvals are presented in (Wegmüller *et al.*, 2020). Children aged 6 - 59 months were recruited from three strata (Southern Belt, Middle Belt, and Northern Belt) in Ghana and random selection was performed in each stratum. Blood sampling and anthropometry were conducted during the survey. Malaria testing was performed using RDT (SD BIOLINE Malaria Ag P.f/Pan RDT kit (Standard Diagnostics Inc, Gyeonggi-do, Republic of Korea). Haemoglobin concentrations were measured using HemoCue 301 AB, Ängelholm, Sweden). Sandwich ELISA (Erhardt *et al.*, 2004) was used to measure serum ferritin and CRP concentrations. DNA was extracted from blood pellets and used for typing sickle cell and α -thalassaemia using PCR at the KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya.

Western Kenya: Children were recruited from the Water, Sanitation, and Handwashing (WASH) Benefits Trial, in rural western Kenya (Byrd *et al.*, 2018). Details of the study design have been published in (Byrd *et al.*, 2018). I used samples collected from the environmental enteropathy endline survey. Venous blood samples were used to test for malaria parasitaemia using RDT (SD BIOLINE Malaria *P. falciparum* [HRP2], Alere, Inc., Waltham, MA) and haemoglobin concentrations (Hemocue Hb 301). Serum ferritin and CRP were assayed using sandwich ELISA (Erhardt *et al.*, 2004). Genotyping of sickle cell and α -thalassaemia was conducted using PCR (Waterfall and Cobb, 2001).

Sud Kivu and Kongo Central, Democratic Republic of Congo: This study used data collected during a nutrition survey of mothers and their children aged 6–59 months in rural Sud Kivu and Kongo Central provinces in the Democratic Republic of Congo (DRC) as described in (Barker *et al.*, 2017). Malaria was tested using RDT (CareStart Malaria Screen, AccessBio, Inc.). Serum ferritin, CRP and AGP were assayed using sandwich ELISA (Erhardt *et al.*, 2004).

Haemoglobin typing was conducted using Pyrosequencing while PCR was used to detect α -thalassaemia and G6PD as described in (Barker *et al.*, 2017).

Muheza, Tanzania: Children were recruited from the *Mother–Offspring Malaria Studies* project at Muheza District Hospital in northeastern Tanzania to investigate iron status and risk of malaria infection as described in (Gwamaka *et al.*, 2012). Blood samples were collected at 3, 6, and 12 months of age then once every 6 months in year 2 and 3 (Gwamaka *et al.*, 2012). In this thesis, I used data from samples collected at a single timepoint, each child's oldest timepoint, since older children were likely to have experienced more malaria episodes. *P. falciparum* parasitaemia was determined using Giemsa-stained thick blood smears while haemoglobin was measured using an impedance-based analyzer (Abbott Cell Dyne 1200). Plasma ferritin and CRP were assayed using ELISA and haemoglobin was typed by electrophoresis (Helena Laboratories, Beaumont, Texas, USA) (Gwamaka *et al.*, 2012). Genotyping for α -thalassaemia was conducted using PCR as detailed in (Chong *et al.*, 2000).

Yaoundé and Douala, Cameroon: Children aged 12-59 months were recruited for a cluster study that aimed to determine the prevalence of inherited haemoglobin disorders in Yaoundé and Douala, Cameroon as described in (Engle-stone *et al.*, 2017). Malaria testing was done using RDT (SD BIOLINE Malaria Ag Pf/Pan, Standard Diagnostics; Gyeonggi-do, Republic of Korea) and haemoglobin was measured using a photometer (Hemocue, Ängelholm, Sweden). Plasma ferritin, CRP and AGP were assayed using ELISA (Erhardt *et al.*, 2004). Haemoglobin genotypes were determined by High Performance Liquid Chromatography (HPLC) using an Ultra-Resolution Variants Analyzer (Trinity Biotech, Bray, Ireland) while α -thalassaemia type was determined by PCR (Engle-stone *et al.*, 2017).

Kilifi hospital-based study: Data from this study were used to determine the levels of hepcidin in 62 children admitted to Kilifi County Hospital with severe malaria. Hepcidin was measured using the Hepcidin-25 [human] Enzyme immunoassay Bachem Kit and harmonised to high sensitive DRG hepcidin values (Wray *et al.*, 2017). Severe malaria was diagnosed as *P. falciparum* parasites in the blood film plus clinical features of severe malaria including haemoglobin <5g/dL or haematocrit level of <15% (for severe malarial anaemia) or Blantyre coma score of <3 (for cerebral malaria).

Jackson Heart Study. This is a population-based cohort study of African-Americans aged ≥ 21 years living in Mississippi, USA (Taylor, 2005). This study was designed to evaluate risks of cardiovascular disease as described in (Taylor, 2005; Wilson *et al.*, 2005). Serum ferritin (Roche immunoturbimetric assay), CRP (ELISA) and haemoglobin (Coulter analyser, Beckman Coulter, USA) were measured from blood samples collected at a single clinic visit. Iron deficiency was defined as ferritin <30 μ g/L, anaemia as haemoglobin >12g/dL in females or <13g/dL in males, iron deficiency anaemia as presence of iron deficiency and anaemia, and inflammation as CRP >5mg/L. Whole blood was used to extract DNA using Puregene reagents (Gentra System, Minneapolis, USA). Genetic studies were conducted as described elsewhere (Wilson *et al.*, 2005). α -thalassemia status was determined using $\sim 30\times$ whole genome sequencing obtained through the NHLBI TOPMed project (<https://www.nhlbiwgs.org/>). Details of the sequencing, variant calling, and quality control protocols are described at dbGaP Study Accession phs000964 (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000964.v4.p1). A subset of 3,009 Jackson Heart Study TOPMed participants underwent genotyping for the α -globin copy number variation using the Genome STRiP multi-sample structural variant calling algorithm (Handsaker *et al.*, 2015).

4.3.2. Effect of sickle cell trait on malaria

I performed a systematic review and meta-analysis of published studies to determine the overall effect of HbAS on uncomplicated malaria. I performed the search in Pubmed using search terms that included (“malaria” [Title/Abstract] OR “malaria/blood” [MAJR] OR “malaria/genetics” [MeSH Terms] OR "Malaria"[Mesh] OR "Malaria, Falciparum"[Mesh] OR "Plasmodium falciparum"[Mesh]) AND (“HbAS” [Title/Abstract] OR “sickle cell trait” [Title/Abstract] OR “sickle cell trait/genetics” [MeSH Terms] OR “sickle cell trait/blood” [MeSH Terms] OR "Haemoglobin, Sickle"[Mesh] OR "Sickle Cell Trait"[Mesh] AND "Africa" [MeSH Terms] on 5th December 2019. I retrieved 585 articles and restricted the analysis to studies conducted in Africa and reporting incidence rate ratio (IRR). Since clinical malaria may occur repeatedly, an IRR provides a better estimate of the true malaria risk reduction attributable to HbAS. I found nine studies which I meta-analysed to obtain an overall IRR of HbAS on the risk of uncomplicated malaria. I used this estimate to weight the effect of HbAS on iron deficiency when calculating the causal estimate in Mendelian randomisation analysis.

4.3.3. Statistical analysis

I conducted all statistical analyses using STATA 13.0 (StataCorp., College Station, TX). I performed both cohort-specific and pooled analyses of the effect of HbAS on iron status. Where appropriate, I fitted unadjusted linear or logistic regression models to determine the effect of HbAS on iron status. Unadjusted analyses are recommended since valid instruments should be independent of potential confounders in the exposure-outcome relationship. I meta-analysed the cohort-specific odds ratios or estimates assuming fixed-effects since there was little evidence of heterogeneity between studies. A P value <0.05 was considered statistically significant. All P-values reflect two-tailed tests

I used the online <http://cnsgenomics.com/shiny/mRnd/> MR power calculator to calculate sample size. The sample size of 7453 had power above 80% given the observed odds ratio of the outcome variable per standard deviation of the exposure variable of 2.52, 10% variation of clinical malaria that is explained by HbAS (Malaria Genomic Epidemiology Network, 2019), 27% of iron deficiency cases and a type-I error rate of 0.05.

I then conducted a two-sample Mendelian randomisation (Evans and Davey Smith, 2015) to investigate whether uncomplicated malaria causes iron deficiency using *mrrobust* software package (Spiller, Davies and Palmer, 2019) in STATA 13.0. This involved two estimates. The first sample estimate was from the community-based cohorts where the overall log-odds of HbAS on iron deficiency was determined using the meta-analysed cohort-specific estimates. The second sample estimate was from the meta-analysed overall log incidence rate of HbAS on uncomplicated malaria. A causal log-odds (equation 1), which is the ratio of the first sample estimate and the second sample estimate was computed (Evans and Davey Smith, 2015). The causal odds ratio was obtained by exponentiating the causal log-odds and interpreted as change in iron deficiency per unit increase in log incidence rate of malaria. I further calculated the effect of reducing malaria incidence by half on iron deficiency. I obtained this by multiplying the causal log-odds by natural-logarithm of 0.5 then exponentiated the result (Burgess and Labrecque, 2018). I repeated the analyses in an African-American population with no exposure to malaria in ‘no relevance point’ sensitivity analyses (Chen *et al.*, 2008).

$$\text{Causal log odds} = \frac{\text{Log odds of iron deficiency in HbAS}}{\text{Log odds of malaria in HbAS}} \quad (1)$$

4.4 Results

4.4.1 Characteristics of study populations

Table 4.1 shows the characteristics of the participants included in these analyses. Studies in malaria-endemic sites included children aged 0 - 8 years while the Jackson Heart Study (JHS), a malaria-free study included adults aged 21 - 95 years. Figure 4.1A shows the study sites mapped against the Africa malaria map of the predicted posterior predictions of age-standardised *P. falciparum* prevalence as previously published by (Snow *et al.*, 2017). The prevalence of malaria parasitaemia, HbAS and iron deficiency in children from the African community-based studies varied across the study sites from 7.26-26.66%, 9.76-16.76% and 10.27-39.46% respectively as shown in Figure 4.1B. In JHS, HbAS was present in 8.43% and iron deficiency in 9.99% of individuals.

Table 4.1. Characteristics of study participants by site

| | Total | Age (years) mean (sd) | Sex: females, n (%) | Inflammation ¹ , n (%) | Stunting ² , n (%) | Underweight ³ , n (%) |
|---|-------|--------------------------|------------------------|--------------------------------------|----------------------------------|-------------------------------------|
| <i>Malaria-exposed studies</i> | | | | | | |
| Malawi | 1035 | 2.75 (1.24) | 512 (49.47) | 604 (58.36) | 319 (31.18) | 134 (13.00) |
| Ghana | 1123 | 2.67 (1.28) | 560 (49.96) | 230 (20.48) | 248 (22.52) | 177 (16.06) |
| Banfora, Burkina Faso | 314 | 1.86 (0.46) | 153 (48.73) | 106 (33.76) | 100 (34.01) | 57 (19.32) |
| Western, Kenya | 411 | 1.85 (0.15) | 210 (51.09) | 92 (22.38) | 95 (23.40) | 32 (7.82) |
| Sud Kivu and Kongo Central, DRC | 678 | 2.41 (1.14) | 338 (49.85) | 462 (68.14) | 329 (53.93) | 141 (22.49) |
| Kilifi, Kenya | 996 | 2.20 (1.47) | 489 (49.10) | 261 (26.20) | 41 (42.71) | 72 (26.67) |
| Muheza, Tanzania | 652 | 1.64 (0.79) | 313 (48.01) | 370 (56.75) | 126 (20.59) | 49 (8.02) |
| West Kiang, The Gambia | 723 | 3.91 (1.16) | 333 (46.06) | 107 (14.80) | 207 (36.51) | 145 (25.53) |
| Yaoundé and Douala, Cameroon | 292 | 2.76 (1.05) | 143 (49.48) | 62 (21.23) | 45 (15.62) | 15 (5.21) |
| Entebbe, Uganda | 1229 | 2.30 (0.82) | 603 (49.06) | 291 (23.68) | 186 (15.33) | 100 (8.16) |
| <i>Malaria-free studies</i> | | | | | | |
| Soweto, South Africa | 648 | 1.02 (0.94) | 317 (48.92) | 100 (15.43) | n/a | n/a |
| Jackson Heart Study | 3207 | 55.57 (12.83) | 2000 (62.36) | 940 (29.31) | n/a | n/a |
| <i>Hospitalised children with severe malaria</i> | | | | | | |
| Kilifi hospital-based study | 62 | 1.86 (1.06) | 28 (45.16) | 62 (100.00) | 21 (38.18) | 26 (41.94) |

DRC, Democratic Republic of Congo; sd, standard deviation; n/a, not available

¹Inflammation was defined as C-reactive protein (CRP) >5mg/L or α_1 -antichymotrypsin (ACT) >0.6g/L or α_1 -acid glycoprotein (AGP) >1g/L. CRP was measured in all sites except West Kiang, The Gambia where only ACT was measured. In addition to CRP, AGP was measured in Malawi, DRC and Cameroon.

²Stunting was defined as a WHO 2006 reference height-for-age z-score <-2

³Underweight was defined as a WHO 2006 reference weight-for-age z-score <-2

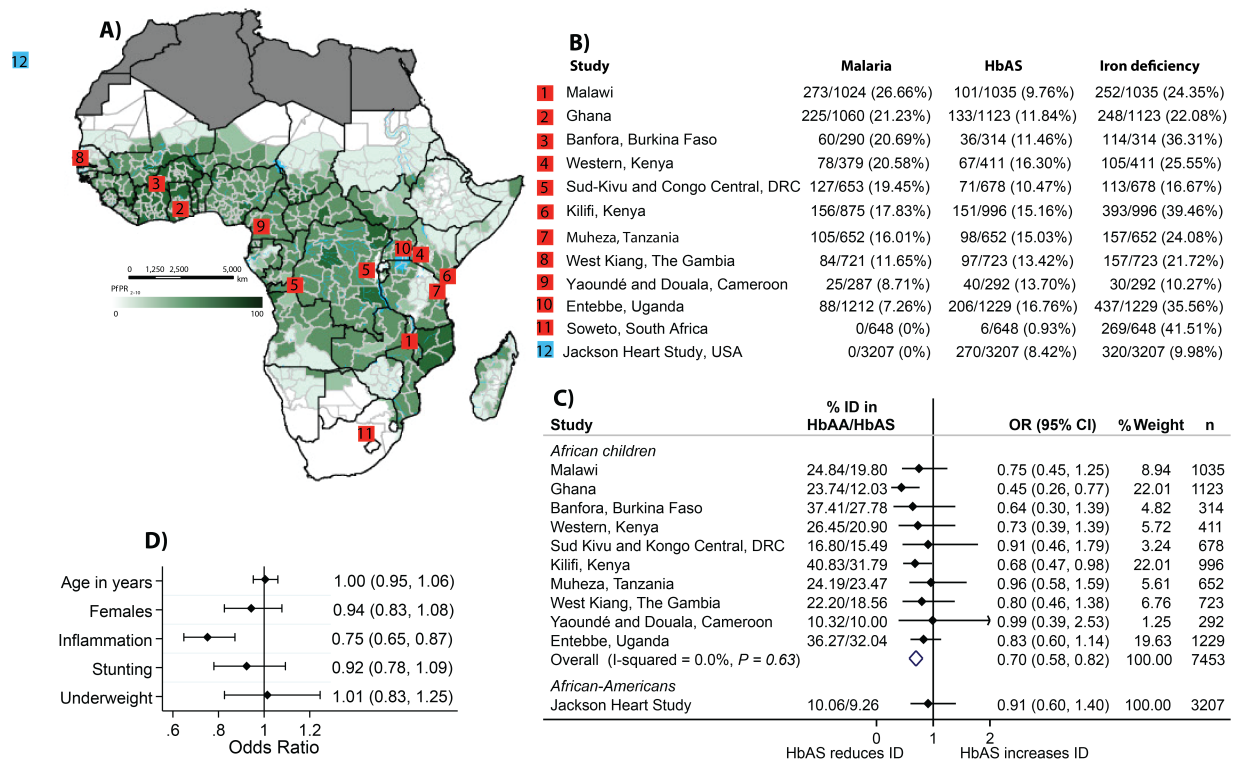


Figure 4.1. Sickle cell trait (HbAS) protects from iron deficiency (ID).

A) Africa malaria map showing the predicted posterior predictions of age-standardised *P. falciparum* prevalence (PfPR₂₋₁₀) as previously published by (Snow et al., 2017) and the current study sites. Map was reproduced with permission. **B)** Prevalence of malaria parasitaemia, sickle cell trait (HbAS), and iron deficiency (ID) by study site. **C)** Summary results of the effect of HbAS on ID by study site, overall fixed-effects meta-analysis of malaria studies, and effect in a non-malaria exposed population, Jackson Heart Study (JHS). The South African cohort was excluded since only 6 out of 648 children had HbAS. Overall represents a fixed-effect meta-analysis of cohort-specific odds ratios. **D)** Associations between sickle cell trait and age, sex, inflammation, stunting and underweight. In malaria studies, ID was defined as plasma ferritin <12µg/L or <30µg/L in the presence of inflammation (CRP >5mg/L or ACT >0.6g/L or AGP >1g/L) in children <5 years old or <15µg/L in children ≥5 years old. In the JHS, ID was defined as plasma ferritin <30µg/L. HbAA, normal haemoglobin.

4.4.2 Sickle cell trait is associated with protection against iron deficiency

I first evaluated the association between HbAS and iron deficiency. In a meta-analysis of the 10 community-based study cohorts of malaria-exposed children ($n = 7453$), HbAS was associated with 30% protection against iron deficiency (OR = 0.70; 0.58, 0.82; $P < 0.001$; Figure 4.1C) and 31% protection against iron deficiency anaemia (OR = 0.69; 0.53, 0.85; $P < 0.001$; Figure 4.2). The protective effect of HbAS against iron deficiency was consistent across cohorts ($I^2 = 0.0\%$, Figure 4.1C). Since HbAS and α -thalassaemia show negative epistatic interaction (Williams, Mwangi, Wambua, Peto, *et al.*, 2005), I further adjusted for the effect of α -thalassaemia on iron deficiency. Adjusting for α -thalassaemia did not alter the effect of HbAS on risk of iron deficiency (Table 4.2). I also obtained similar results in pooled analyses compared to the meta-analysis (OR = 0.79 (0.68, 0.93); $P = 0.003$). Prevalence of iron deficiency and iron deficiency anaemia were similarly lower in malaria-exposed individuals carrying HbAS compared to those carrying HbAA (Figures 4.3A and 4.3B). Since poor nutrition could also lead to iron deficiency, I tested whether HbAS might be associated with stunting or underweight and found little evidence of an association (Figure 4.1D). HbAS was similarly not associated with age or sex ($P > 0.05$; Figure 4.1D). In sensitivity analyses, HbAS was not associated with iron deficiency in non-malaria exposed African-Americans (OR = 0.91; 0.60, 1.40; $P = 0.68$; Figure 4.1C and Figure 4.3A) suggesting that HbAS is protective against iron deficiency only in malaria-exposed populations.

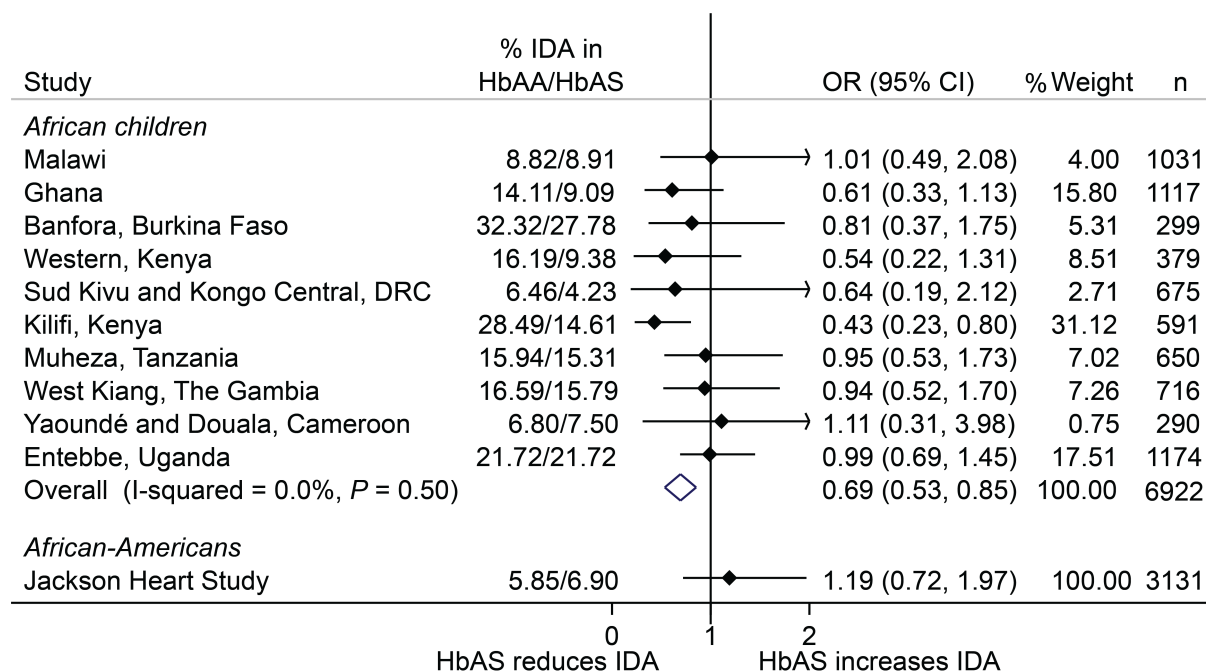


Figure 4.2. A meta-analysis of the effect of sickle cell trait on iron deficiency anaemia (IDA).

Overall represents a fixed-effect meta-analysis of cohort-specific odds ratios. *n* shows the number of individuals included in the analysis.

Table 4.2. Effect of sickle cell trait (HbAS) on iron deficiency before and after adjusting for α -thalassaemia.

| Study | HbAS | | HbAS adjusted for α -thalassaemia | |
|---------------------------------------|------|-------------------|--|-------------------|
| | n | OR (95% CI) | n | OR (95% CI) |
| Malawi | 1035 | 0.75 (0.45, 1.25) | 1030 | 0.76 (0.45, 1.26) |
| Ghana | 1123 | 0.45 (0.26, 0.77) | 1045 | 0.46 (0.26, 0.80) |
| Western, Kenya | 411 | 0.73 (0.39, 1.39) | 409 | 0.73 (0.38, 1.38) |
| Sud Kivu and Kongo Central, DRC | 678 | 0.91 (0.46, 1.79) | 381 | 0.66 (0.22, 1.98) |
| Kilifi, Kenya | 996 | 0.68 (0.47, 0.98) | 991 | 0.68 (0.47, 0.98) |
| Muheza, Tanzania | 652 | 0.96 (0.58, 1.59) | 616 | 0.99 (0.59, 1.70) |
| Yaoundé and Douala, Cameroon | 292 | 0.99 (0.39, 2.53) | 270 | 0.99 (0.32, 3.08) |
| Overall (fixed-effects meta-analysis) | 4046 | 0.76 (0.58, 0.93) | 3697 | 0.74 (0.56, 0.92) |

Summary results of the effect of HbAS on iron deficiency by study site, overall fixed-effects meta-analysis of children in malaria-exposed cohorts, and effect of HbAS on iron deficiency after adjusting for α -thalassaemia.

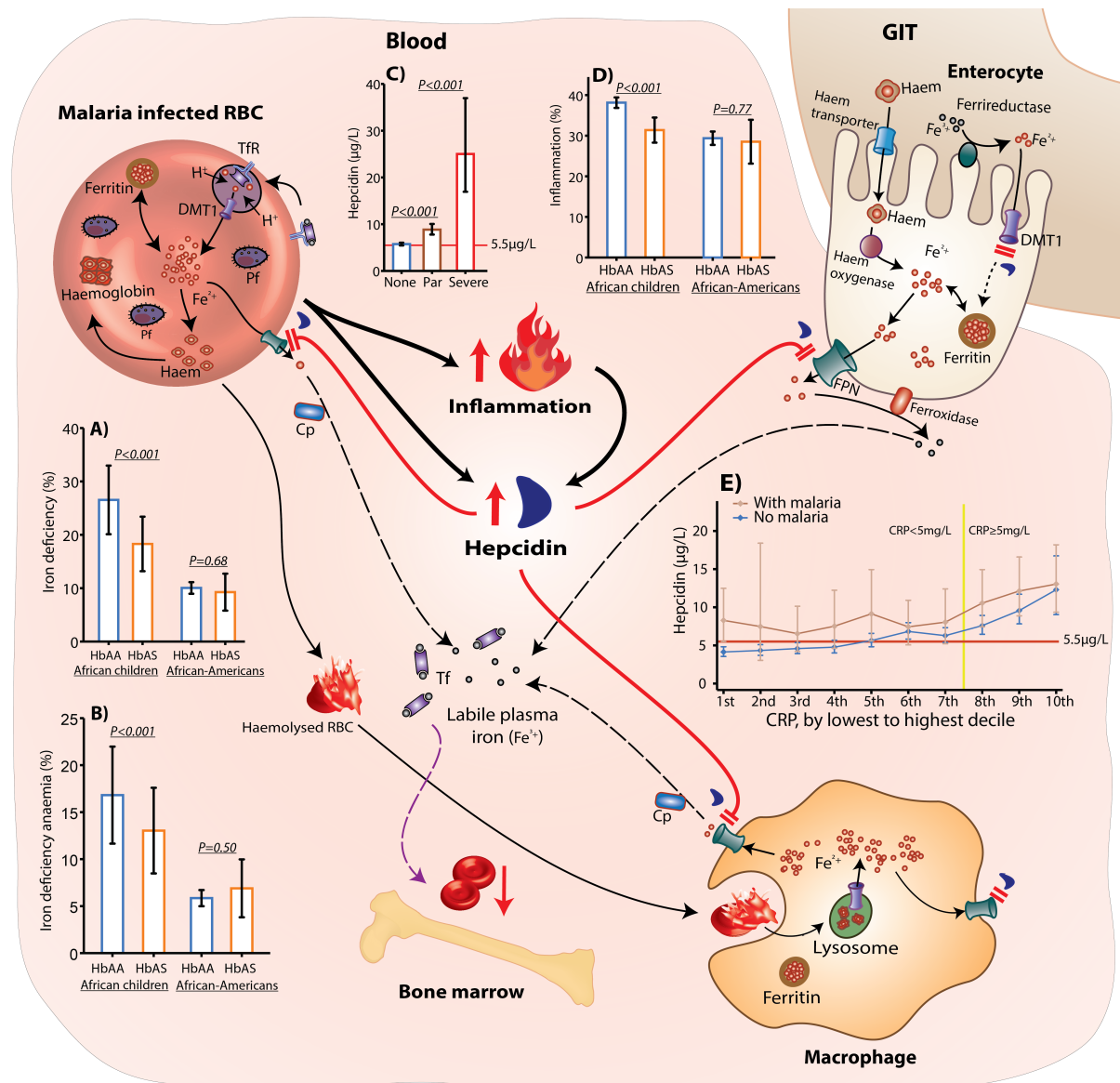


Figure 4.3. Malaria may cause iron deficiency through hepcidin-mediated blockade of iron absorption and recycling.

A) Prevalence of iron deficiency is lower in children carrying sickle cell trait (HbAS) than in those with normal haemoglobin (HbAA) in malaria studies but not in the Jackson Heart Study (JHS), a malaria-free population. **B)** Lower prevalence of iron deficiency anaemia in children carrying HbAS in malaria studies, but not in JHS. **C)** Higher hepcidin concentrations in children with malaria parasitaemia or severe malaria compared to those without. **D)** The prevalence of inflammation is lower in HbAS individuals compared to those carrying normal haemoglobin (HbAA) in malaria studies but not in JHS. **E)** Hepcidin concentrations increase with increasing CRP concentrations and remain above the threshold for iron absorption at all deciles of CRP in children with malaria parasitaemia. Yellow line shows point at which inflammation is clinically diagnosed. Red horizontal line indicates the threshold of hepcidin above which iron absorption is inhibited (5.5µg/L). Cp, Ceruloplasmin; DMT1, divalent metal transporter 1; FPN, ferroportin; GIT, gastrointestinal tract; Par, malaria parasitaemia; Pf, *P. falciparum*; Tf, transferrin; TFR, transferrin receptor. This diagram is my own illustration.

Having shown that HbAS is associated with iron deficiency in African children, but not in African Americans, I then estimated the causal effect of uncomplicated malaria on iron deficiency using HbAS as an instrumental variable in a two-sample Mendelian randomisation analysis (Davey Smith and Hemani, 2014). This requires weighting the effect of HbAS on iron deficiency using the effect of HbAS on uncomplicated malaria. The effect of HbAS on severe malaria can be obtained from genome-wide association studies (GWAS), however, there are no previous GWAS of uncomplicated malaria. I used the effect of HbAS on uncomplicated malaria since severe malaria is a rare outcome and thus less likely to be a major cause of iron deficiency at the population level. I therefore conducted a systematic review and meta-analysis of all published papers that assessed the effect of HbAS on the incidence of uncomplicated malaria to obtain an overall incidence rate ratio (Figure 4.4 and Table 4.3). Overall, HbAS was associated with 32% (IRR = 0.68; 0.62, 0.73) protection from episodes of uncomplicated malaria (Figure 4.4).

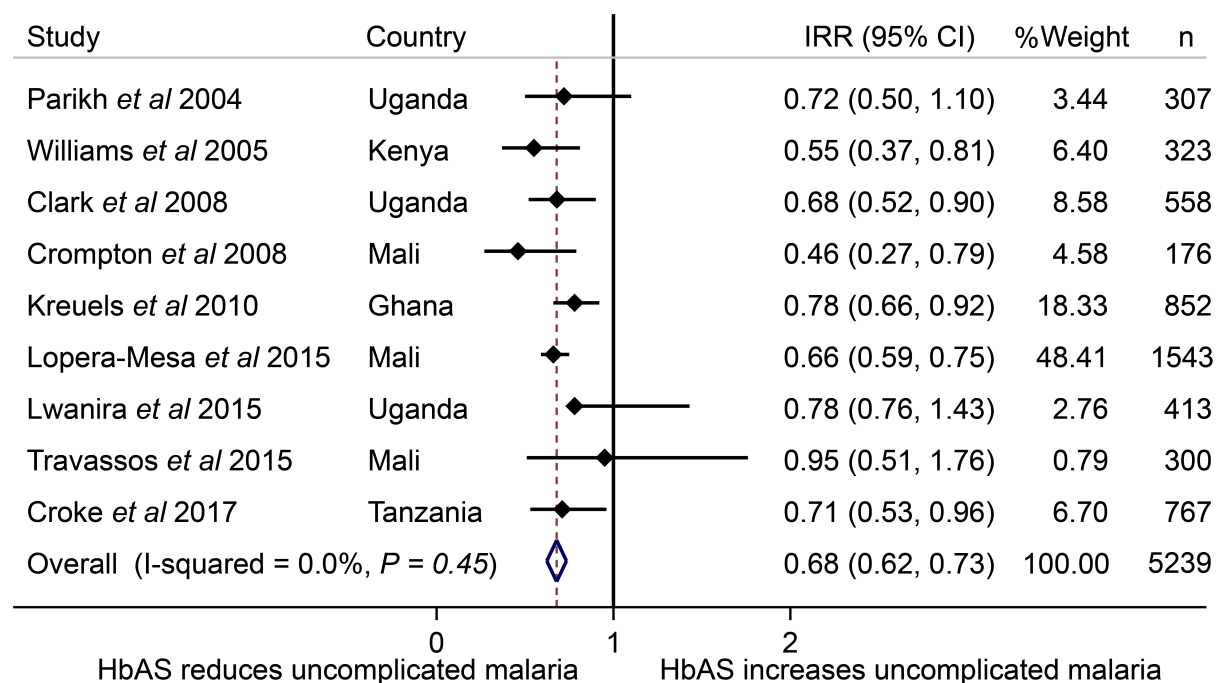


Figure 4.4. A meta-analysis of the effect of sickle cell trait on uncomplicated malaria. Overall represents a fixed-effect meta-analysis of study-specific incidence rate ratio. *n* shows the number of individuals included in the analysis.

Table 4.3. A summary of published studies on the incidence rate ratio of sickle cell trait on uncomplicated malaria

| Study | Country | Sample size | Age | IRR (95% CI) |
|---|----------|-------------|---------------------|-------------------|
| (Parikh, Dorsey and Rosenthal, 2004) | Uganda | 307 | 6 months - 5 years | 0.72 (0.50, 1.10) |
| (Williams, Mwangi, Wambua, Alexander, <i>et al.</i> , 2005) | Kenya | 323 | <8 years | 0.55 (0.37, 0.81) |
| (Clark <i>et al.</i> , 2008) | Uganda | 558 | 1-10 years | 0.68 (0.52, 0.90) |
| (Crompton <i>et al.</i> , 2008) | Mali | 176 | 2-10 years | 0.46 (0.27, 0.79) |
| (Kreuels <i>et al.</i> , 2010) | Ghana | 852 | 3 months - 2 years | 0.78 (0.66, 0.92) |
| (Lopera-Mesa <i>et al.</i> , 2015) | Mali | 1543 | 6 months - 17 years | 0.66 (0.59, 0.75) |
| (Lwanira <i>et al.</i> , 2015) | Uganda | 413 | 3 - 9 years | 0.78 (0.76, 1.43) |
| (Travassos <i>et al.</i> , 2015) | Mali | 300 | 0 - 6 years | 0.95 (0.51, 1.76) |
| (Croke <i>et al.</i> , 2017) | Tanzania | 767 | 0 - 19 years | 0.71 (0.53, 0.96) |

I then calculated the causal estimate in a two-sample Mendelian randomisation analysis (Davey Smith and Hemani, 2014). I estimated the causal log odds estimate as the ratio of the log odds of HbAS on iron deficiency (Figure 4.1C) to log incidence rate of HbAS on uncomplicated malaria (Figure 4.4). The betas and standard errors are shown in Table 4.4. I exponentiated the causal log odds (0.92), to obtain the causal odds ratio. I observed a causal odds ratio of 2.52 (95% CI 1.60, 3.98; $P < 0.001$) suggesting that genetically predicted risk of uncomplicated malaria is associated with 2.5-fold higher odds of iron deficiency per unit increase in log incidence rate of malaria. Thus, reducing the incidence of uncomplicated malaria by half would reduce iron deficiency by 47% (that is, odds of 0.53 calculated as exponentiate ($\log 0.5 \times 0.92$)).

Table 4.4. Log estimates used in Mendelian randomisation analyses

| Relation | Beta (SE) | P | Source |
|------------------------------|--------------|--------|---|
| HbAS - ID | -0.36 (0.09) | <0.001 | Meta-analysed African studies (Fig. 4.1C) |
| HbAS - uncomplicated malaria | -0.39 (0.05) | <0.001 | Meta-analysis of published studies (Fig. 4.4) |
| MR causal estimate | 0.92 (0.23) | <0.001 | Calculated as HbAS-ID/HbAS-malaria |

SE, standard error; HbAS, sickle cell trait; ID, iron deficiency; MR, Mendelian randomisation

I compared the findings for HbAS to other genetic polymorphisms associated with protection against malaria by using data for 5021 and 4811 children with G6PD deficiency and α -

thalassaemia carriage data respectively (Figures 4.5 and 4.6). These polymorphisms are mainly associated with protection from severe malaria. However, in previous studies G6PD deficiency was associated with 30% protection against uncomplicated malaria in heterozygous females (Mbanefo *et al.*, 2017), while α -thalassaemia may protect from uncomplicated malaria in heterozygotes (Enevold *et al.*, 2008). Overall, I found that heterozygous G6PD females and heterozygous α -thalassaemic children were 39% (OR 0.61; 0.46, 0.76) and 19% (OR 0.81; 0.68, 0.95) protected from iron deficiency thus supporting the HbAS data. However, findings were less consistent across cohorts. These polymorphisms were not associated with iron deficiency in non-malaria exposed African-Americans (Figures 4.5 and 4.6).

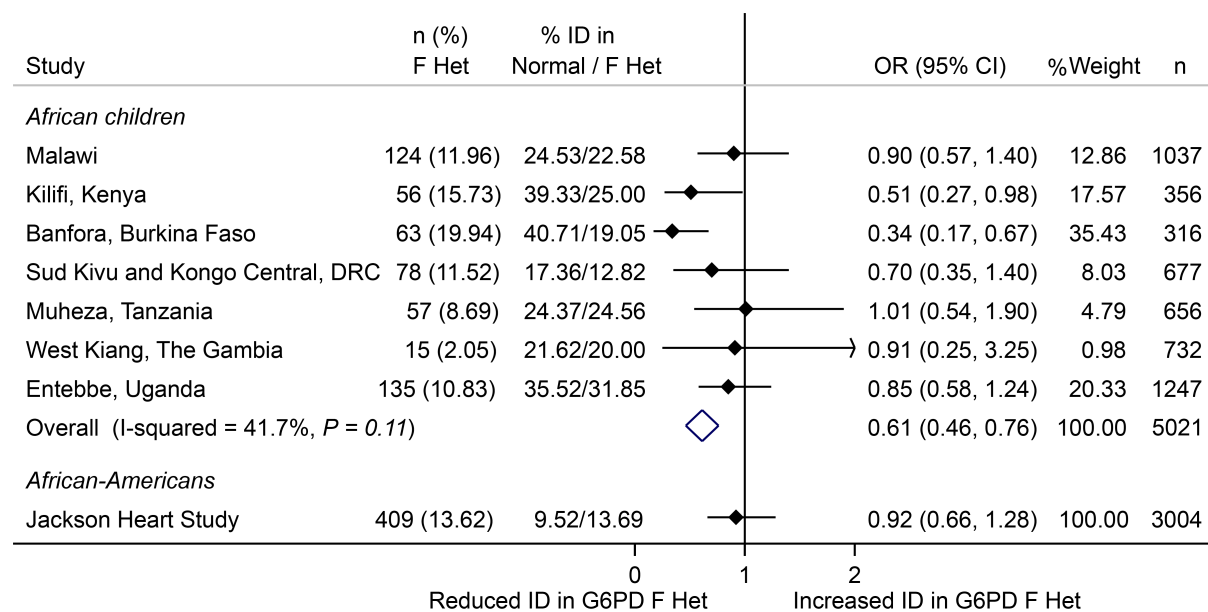


Figure 4.5. A meta-analysis of the effects of glucose-6-phosphate-dehydrogenase deficiency (G6PD) heterozygosity on iron deficiency.

Overall represents a fixed-effect meta-analysis of cohort-specific odds ratios. n shows the number of individuals included in the analysis. F Het, female heterozygous; ID, iron deficiency.

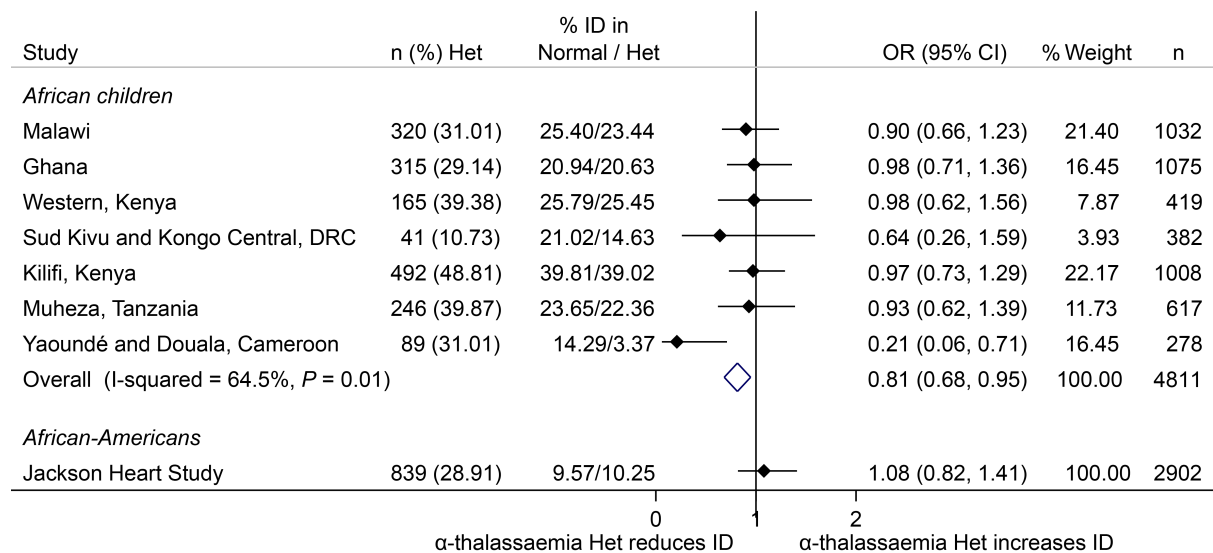


Figure 4.6. A meta-analysis of the effects of alpha-thalassaemia heterozygosity on iron deficiency.

Overall represents a fixed-effect meta-analysis of cohort-specific odds ratios. n shows the number of individuals included in the analysis. Few individuals were homozygous for alpha-thalassaemia and were excluded from the analysis. Het, heterozygous; ID, iron deficiency.

4.4.3 Malaria may cause iron deficiency through a hepcidin-mediated blockade of iron absorption and recycling

I hypothesised that malaria may be causing iron deficiency *via* a hepcidin-mediated blockade of iron absorption and recycling, as illustrated in Figure 4.3 above. I observed that children with asymptomatic malaria parasitaemia and severe malaria had increased mean hepcidin concentrations compared to those without malaria (Figure 4.3C). Moreover, mean hepcidin concentrations in children with either asymptomatic malaria parasitaemia or severe malaria were well above the threshold of $5.5\mu\text{g/L}$ associated with iron blockade (Prentice *et al.*, 2012; Pasricha *et al.*, 2014). Since anaemia suppresses hepcidin production (Casals-Pascual *et al.*, 2012), I further stratified the effects of malaria on hepcidin levels by anaemia status and found that hepcidin levels were below a threshold associated with iron blockade only in individuals with anaemia and without malaria parasitaemia (Figure 4.7). Children with asymptomatic

malaria parasitemia, and severe malaria, had higher geometric mean CRP levels (4.62 mg/L, and 100.28 mg/L, respectively) compared to those without malaria (1.15 mg/L).

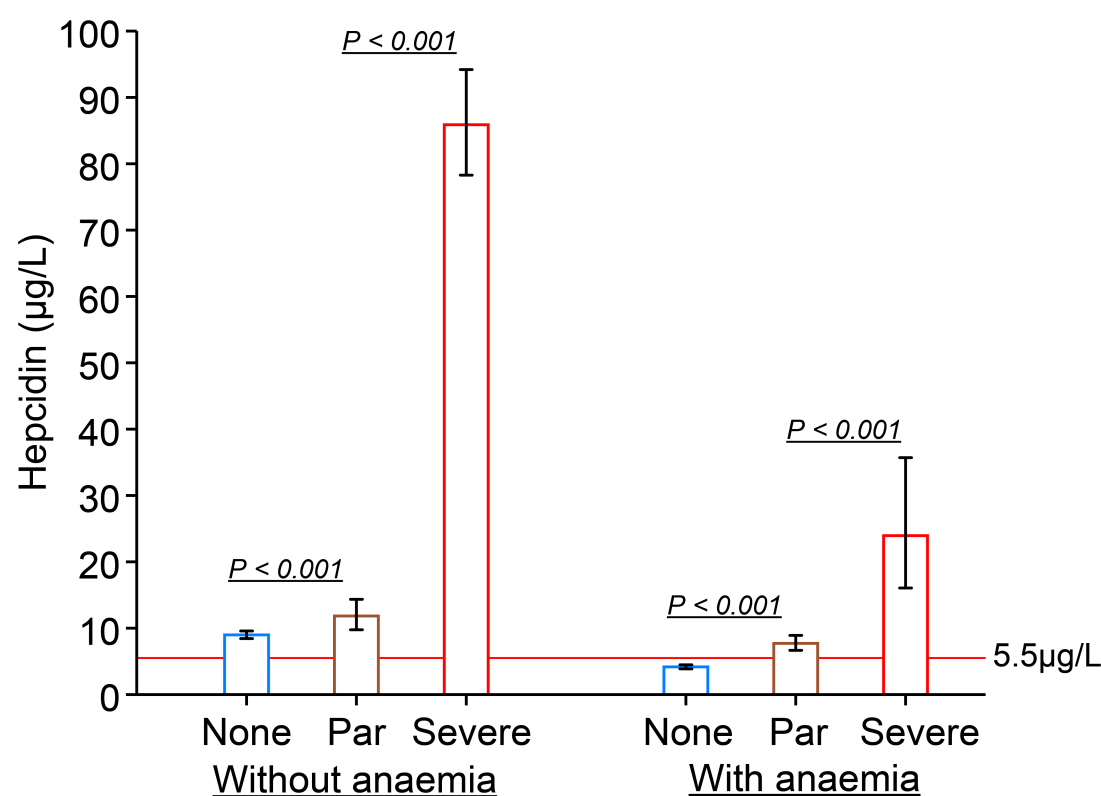


Figure 4.7. Geometric mean hepcidin concentrations by anaemia status.

Par, malaria parasitaemia; Severe, severe malaria

Since inflammation also increases hepcidin levels, I also analysed the interaction between malaria and inflammation in influencing hepcidin concentrations. I found that HbAS was associated with a 25% reduction in inflammation (OR 0.75; 0.65, 0.87; $P < 0.001$; Figures 4.2D and 4.3D) in malaria-exposed African children but not in a non-malaria exposed population of African-Americans (OR 0.96; 0.73, 1.26; $P = 0.77$; Figure 4.3D). I further observed that hepcidin concentrations were raised above a threshold shown to block iron absorption, even at very low CRP concentrations (1mg/L, Figure 4.3E) long before children would be classified as having inflammation (C-reactive protein (CRP) ≥ 5 mg/L). Notably, children with malaria parasitaemia had higher hepcidin concentrations at almost every decile of CRP compared to children without parasitaemia (Figure 4.3E). However, HbAS itself was not associated with hepcidin concentrations (log estimate = 0.07; 95% CI -0.05, 0.20; $P = 0.24$; $n = 3473$).

4.5 Discussion

The causal relationship between malaria and iron deficiency is complex and difficult to disentangle in observational studies due to confounding bias and reverse causality. Here, I applied Mendelian randomisation, an approach that overcomes some of the limitations of observational studies by utilising sickle cell trait to proxy malaria exposure. Using large-scale data ($n = 11,370$) from children across the African continent and from African-Americans I found that uncomplicated malaria is causally associated with an increased risk of iron deficiency in African children. These findings suggest that an intervention that halves the incidence of uncomplicated malaria episodes would reduce prevalence of iron deficiency by 47%.

My findings corroborate reports from previous interventional and observational studies. Treatment of afebrile malaria has been shown to reduce hepcidin concentrations by approximately half, double iron absorption and reduce iron deficiency by approximately half in Ivorian children (Glinz, Hurrell, Righetti, *et al.*, 2015). Similar results have been reported in Beninese women treated for afebrile malaria (Cercamondi *et al.*, 2010). A study in Kenyan and Gambian children reported significant increase in prevalence of iron deficiency at the end of a malaria season compared to the start (from 38.2% to 46.5% in Kenyans and from 18.6% to 29.5% in Gambians) suggesting that malaria may contribute to iron deficiency (Atkinson *et al.*, 2014). Similarly, in another study in the Kenyan highlands, interruption of malaria transmission using antimalarials and indoor residual spraying reduced prevalence of iron deficiency (from 35.6% to 24.9%) and more than halved the prevalence of iron deficiency anaemia (from 27.2% to 12.2%) (Frosch *et al.*, 2014). Thus, the predicted benefits from my analyses of reducing iron deficiency by approximately half through application of an intervention that halves malaria episodes may be translatable to the real world.

Malaria control can be achieved by controlling vectors with insecticide-treated bed nets (ITNs) and Indoor Residual Spraying (IRS), anti-malarial drugs and larval habitat control. Vaccination is a possibility in the future with the RTSS vaccine having an approximately 30% efficacy (Bejon *et al.*, 2008; The RTSS Clinical Trials Partnership, 2014). ITNs, IRS and artemisinin combination therapies have been associated with approaching 50% reductions in the burden of clinical malaria (Bhatt *et al.*, 2015). Furthermore, seasonal malaria chemoprevention or intermittent preventative treatment in children has been associated with protective efficacy of up to 75% against clinical malaria, and is safe and cost-effective (Aponte *et al.*, 2009; Wilson, 2011; Meremikwu *et al.*, 2012). I therefore speculate that a scale-up of these malaria control strategies could achieve more than 47% reduction in iron deficiency among African children with substantial public health benefits.

Malaria may be causing iron deficiency in African children through chronically up-regulating production of hepcidin. In agreement with previous studies, I found that children with asymptomatic malaria infection had increased hepcidin concentrations (de Mast *et al.*, 2010; Atkinson *et al.*, 2015). Interestingly, I found that hepcidin concentrations in parasitaemic children were well above a threshold of 5.5 $\mu\text{g/L}$ beyond which iron absorption has been shown to be impaired (Prentice *et al.*, 2012; Pasricha *et al.*, 2014). I then tested whether the effect of malaria on hepcidin might be mediated by inflammation since IL-6 strongly up-regulates hepcidin production (Nemeth, Rivera, *et al.*, 2004; Atkinson *et al.*, 2014; Prentice *et al.*, 2019). I found that sickle cell trait was associated with reduced inflammation in malaria-exposed African children but not in a non-malaria exposed population of African-Americans. This finding suggests that malaria may be an important cause of inflammation in African children.

Malaria raised hepcidin concentrations above a threshold associated with iron blockade (Prentice *et al.*, 2012; Pasricha *et al.*, 2014) and at CRP levels below a threshold defining inflammation suggesting that malaria may also increase hepcidin concentrations via non-inflammatory pathways (Spottiswoode *et al.*, 2017). Malaria might also cause hepcidin-mediated blockade of iron by increasing the prevalence of bacterial infections (Scott *et al.*, 2011) or by increasing inflammatory cytokines such as TNF α and IFN γ (Atkinson *et al.*, 2008). Possible pathways mediating the relationship between malaria and iron deficiency are shown in Figure 4.8. These findings support previous small radioisotope studies showing that treating malaria reduces hepcidin concentrations, increases iron absorption, and reduces prevalence of iron deficiency (Cercamondi *et al.*, 2010; Glinz, Hurrell, Righetti, *et al.*, 2015).

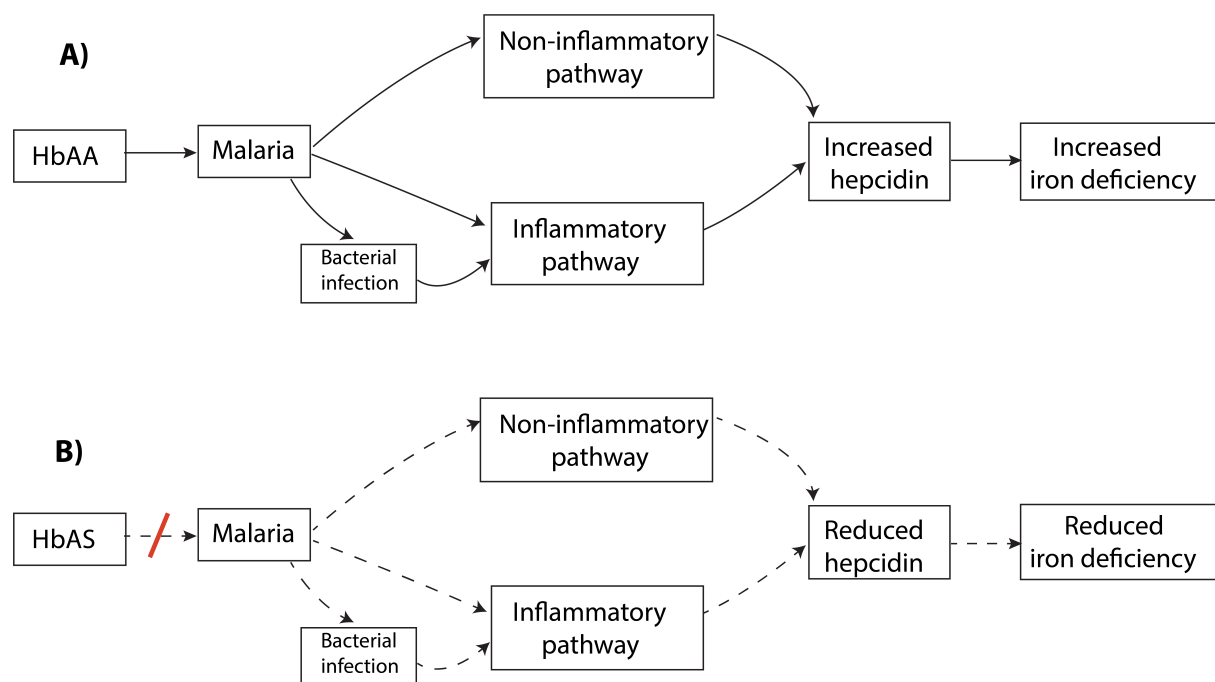


Figure 4.8. How sickle cell trait, a proxy for reduced malaria exposure, may be protecting from iron deficiency.

A) Individuals carrying normal beta haemoglobin gene (HbAA) are not protected from malaria. Malaria up-regulates production of hepcidin through inflammatory and non-inflammatory pathways and by increasing the prevalence of other infections. Hepcidin in turn blocks iron absorption. **B)** Sickle cell trait (HbAS) partially protects individuals from malaria infection therefore inflammation is reduced leading to lower levels of hepcidin and increased iron absorption and recycling.

To test for direct effects of sickle cell trait on iron deficiency (through mechanisms independent of malaria exposure), I repeated the analyses in a non-malaria population using no-relevance point sensitivity analyses (Chen *et al.*, 2008) and also tested effect of sickle cell trait on potential confounders. Sickle cell trait was not associated with iron deficiency or inflammation in African-Americans, a population that is not exposed to malaria. I also found that sickle cell trait was not associated with age, sex, or nutritional status. This suggests that the protective effect of sickle cell trait on iron deficiency in malaria-exposed African children is likely to act through malaria.

Other polymorphisms have also been shown to protect against malaria. In a meta-analysis, heterozygous G6PD deficiency in females conferred 30% protection from uncomplicated malaria (Mbanefo *et al.*, 2017). Although there are mixed findings (Taylor, Parobek and Fairhurst, 2012), heterozygous α -thalassaemia may protect against uncomplicated malaria (Enevold *et al.*, 2008). I found that these polymorphisms were associated with protection from iron deficiency. However, G6PD and α -thalassaemia largely protect from severe malaria, a rare outcome, rather than uncomplicated malaria which, due to its high incidence, is more likely to cause iron deficiency at the population level. Nevertheless, these findings support the results obtained using sickle cell trait, which has been shown to consistently and strongly protect from uncomplicated malaria (Taylor, Parobek and Fairhurst, 2012).

These analyses had a number of limitations. One limitation was the use of African-American adults rather than African children to test whether sickle cell trait might influence iron deficiency through a non-malaria related pathway (pleiotropy). I was unable to test for pleiotropy in non-malaria-exposed South African children since few carried sickle cell trait ($n = 6/648$), similar to most populations without historical exposure to malaria. However, I

obtained similar results using G6PD deficiency and α -thalassaemia, which protect against malaria *via* different mechanisms. Another limitation is that I did not have data on additional potential causes of iron deficiency in the various populations, such as differences in diet, risk of hookworm infection, or other infections such as human immunodeficiency virus. Nevertheless, I obtained a consistent protective effect of sickle cell trait on iron deficiency across all cohorts. I would expect sickle cell trait to have a larger protective effect against iron deficiency in populations with higher incidence of clinical malaria (Spiller *et al.*, 2019), but I did not have data on incidence of clinical malaria for the majority of the cohorts. It is also likely that my analyses have underestimated the effect of malaria on iron deficiency since sickle cell trait is not known to protect against afebrile malaria parasitaemia (Taylor, Parobek and Fairhurst, 2012), prevalent in approximately a quarter of African children (Snow *et al.*, 2017). Afebrile parasitaemia has been shown to increase hepcidin concentrations, in agreement with my analyses, and thus impair iron absorption (Cercamondi *et al.*, 2010; Glinz, Hurrell, Righetti, *et al.*, 2015).

4.6 Conclusion

Using large-scale data from across the malaria-endemic region of sub-Saharan Africa, I have shown that uncomplicated malaria increases risk of iron deficiency in African children. This finding has important policy implication and represent an important shift in the paradigm of iron and malaria infection. Studies to date have largely focused on iron status as a risk factor for malaria, and the difficulties this causes for strategies to treat iron deficiency. These findings suggest that malaria itself may be causing iron deficiency in African children. The management of iron deficiency has traditionally involved iron supplementation, but there are long-standing concerns regarding safety since iron may increase the risk of infections such as malaria (Sazawal *et al.*, 2006) and alter the gut microbiome (Paganini and Zimmermann, 2017).

Moreover, iron supplementation may be ineffective in malaria-endemic areas due to poor absorption in children with chronically raised hepcidin from malaria parasitaemia and other infections (Glinz, Hurrell, Righetti, *et al.*, 2015; Prentice *et al.*, 2019), and treatment of malaria would allow iron to be more effectively absorbed from the diet. Thus, malaria treatment and prevention should be an integral part of programs to control iron deficiency in African children.

CHAPTER FIVE: IRON STATUS AND ASSOCIATED MALARIA RISK AMONG AFRICAN CHILDREN

The material in this chapter forms the basis of a published paper in *Clinical Infectious Diseases* entitled “*Iron Status and Associated Malaria Risk Among African Children*”, in which I was the first author.

5.1 Abstract

Introduction: It remains unclear whether improving iron status increases malaria risk, and few studies have looked at the effect of host iron status on subsequent malaria infection. I therefore aimed to determine whether a child’s iron status influences their subsequent risk of malaria infection in Kenyan and Ugandan children.

Methods: I used data on iron and inflammatory biomarkers and surveillance of malaria episodes from community-based cohorts of 1309 Kenyan and 1374 Ugandan children aged 0 - 7 years. I fitted Poisson regression and Cox proportional hazards models to determine the effect of iron status on the incidence rate ratio of malaria using longitudinal data covering a period of six months. I adjusted for age, sex, malaria parasitaemia, inflammation and study site in the models.

Results: The risk of malaria was 33% and 28% lower in children with iron deficiency (incidence rate ratio, IRR = 0.67; 95% CI: 0.55, 0.82; $P < 0.001$) and iron deficiency anaemia (IRR = 0.72; 95% CI: 0.56, 0.93; $P = 0.006$), respectively. Low transferrin saturation (<10%) was similarly associated with lower risk of malaria (IRR = 0.79; 95% CI: 0.65, 0.96; $P = 0.016$). However, variation in hepcidin, soluble transferrin receptors (sTfRs) and haemoglobin / anaemia was not associated with altered malaria risk.

Conclusions: Iron deficiency appears to protect against malaria infection in African children when defined using ferritin and transferrin saturation, but not when defined by hepcidin, sTfR or haemoglobin. Further research is required to confirm causality and determine mechanisms.

5.2 Introduction

The safety of iron supplementation is a long-standing concern among policy makers and clinicians in malaria-endemic areas (Suchdev *et al.*, 2010; Brittenham, 2012). In Chapter One Section 1.3.3, I reviewed the literature on iron status and malaria risk. Clinical trials have reported conflicting findings on whether iron supplementation influence risk of malaria (Menendez *et al.*, 1997; Sazawal *et al.*, 2006; Veenemans *et al.*, 2011; Esan *et al.*, 2013; Zlotkin *et al.*, 2013). Furthermore, previous randomised controlled trials have reported conflicting findings on whether baseline iron status of a child determines their risk of malaria infection following iron supplementation (Sazawal *et al.*, 2006; Veenemans *et al.*, 2011; Zlotkin *et al.*, 2013). Thus, it is unclear whether iron supplementation might be unsafe because it improves iron status itself thus resulting in a long-term increase in the risk of malaria.

Five observational studies have investigated the effect of a child's iron status on malaria risk. These studies all indicate that iron deficiency is associated with a lower risk of *P. falciparum* malaria in African children (Nyakeriga *et al.*, 2004; Gwamaka *et al.*, 2012; Jonker *et al.*, 2012; Barffour *et al.*, 2017; Moya-Alvarez *et al.*, 2017). However, these studies have largely defined iron deficiency using ferritin only despite its limitations and the availability of other commonly measured iron markers. Thus, little is known about whether other indicators of iron status influence malaria risk in humans. These other indicators of iron status include hepcidin, haemoglobin, soluble transferrin receptors (sTfR), and transferrin saturation (TSAT). Hepcidin has been shown to play a role in preventing superinfection by depriving the *Plasmodium* liver

stage of iron in mice models (Portugal *et al.*, 2011). However, there are mixed findings on whether hepcidin protects from malaria risk in children (Atkinson *et al.*, 2015; Brickley *et al.*, 2016). In Kenyan children, hepcidin concentrations were not associated with malaria risk (Atkinson *et al.*, 2015) while high cord blood hepcidin was associated with lower risk of uncomplicated malaria in Tanzanian infants (Brickley *et al.*, 2016). Two previous observational studies have reported that haemoglobin concentrations do not influence malaria risk (Ghosh *et al.*, 1995; Lombardo *et al.*, 2017) while *in vitro* culture indicates otherwise (Goheen *et al.*, 2016). There are no specific reports of the influence of sTfR and TSAT on malaria in humans. In this chapter, I report the largest observational study on iron status and risk of malaria to date with the most comprehensive range of iron markers in 2683 Kenyan and Ugandan children.

5.3 Methods

5.3.1 Study population

In this chapter, I report analyses of iron / inflammatory markers and malaria episode data from the Kilifi, Kenya and Entebbe, Uganda cohorts described in Chapter Two. I included these two studies since they were the only cohorts with available longitudinal data of active malaria surveillance. Longitudinal parasitaemia data were obtained from active surveillance during the six months following measurement of iron and inflammatory biomarkers (Figure 5.1). In Kenya, children were followed up weekly while in Uganda follow-up involved fortnightly home visits and quarterly clinic visits. Almost all children were followed up for the full six months period (94% in Kenya and 90% in Uganda) and the length of follow-up for the remainder ranged from 1-5 months. I chose a follow-up period of not more than six months since iron status may change over a longer follow-up period. However, I conducted secondary analyses that included a one-year period of follow-up. Clinical malaria data included

microscopy confirmed density of asexual *P. falciparum* parasitaemia and temperature. A malaria episode was defined as parasitaemia at any density and temperature above 37.5°C. All uncomplicated malaria episodes occurring during the follow-up period were included except those occurring within 14 days of an initial presentation, which were regarded as recrudescence.

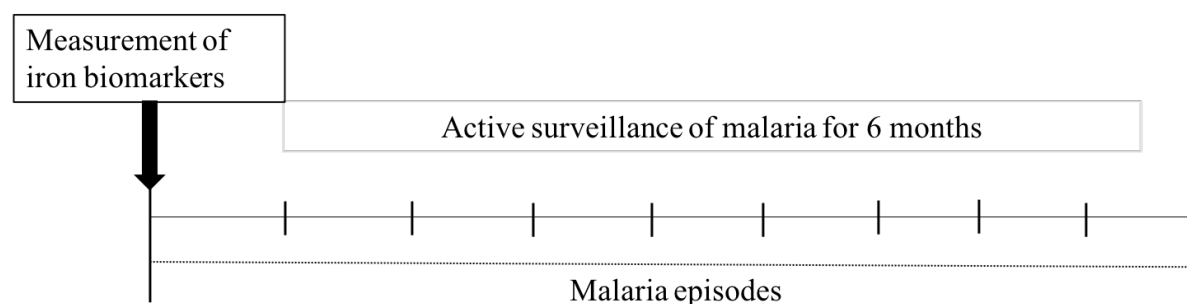


Figure 5.1. Study design.

Iron measurements were taken at baseline. Kenyan children were followed up weekly. In Uganda, follow-up involved fortnightly home visits and quarterly clinic visits.

5.3.2 Statistical analysis

I conducted all analyses using STATA 13.0 (StataCorp., College Station, TX). I ln-transformed iron biomarkers (except haemoglobin) to normalise their distributions. I then computed the geometric means of iron biomarkers and proportions of iron deficiency and anaemia. I used two-tailed Student's *t*-tests to test for difference in means between groups. I fitted Poisson regression models of counts of malaria episodes as predicted by iron status (iron deficiency / anaemia / individual iron biomarkers) adjusting for potential confounders including age, sex, malaria parasitaemia, inflammation and study site. I accounted for difference in individual length of follow-up by including the length of follow-up as “exposure” in the model. I accounted for multiple episodes using robust cluster variance estimation which takes into account correlations between multiple events. Secondary analyses involved excluding children with parasitaemia or inflammation at baseline to mitigate the effects of concurrent infection on

iron status (Aguilar *et al.*, 2012). I used Cox proportional hazards analyses to evaluate the temporal effect of iron status on malaria risk. A p-value of <0.05 was considered significant.

I finally searched the databases PubMed and Google Scholar with search terms that included “iron deficiency or ferritin or hepcidin or sTfR or TSAT or haemoglobin or anaemia and malaria children”. I found five longitudinal studies investigating the effect of iron deficiency on malaria risk but only four reported effect sizes. I then performed a meta-analysis of the current study and the previous longitudinal studies investigating the relationship between iron deficiency and malaria risk using the “*metan*” command in STATA.

5.4 Results

5.4.1 Baseline characteristics of study population

I included a total of 1309 Kenyan and 1374 Ugandan children aged between 0 – 7 years and 1 – 5 years respectively in the analyses. Table 5.1 shows the characteristics of study participants. At baseline, the prevalence of iron deficiency and iron deficiency anaemia were 36.9% and 23.6% in Kenyan and 34.6% and 17.6% in Ugandan children, respectively. The prevalence of iron deficiency based on TSAT (measured in Kenya only) was 52.4%. The prevalence of malaria parasitaemia was higher in Kenyan (20.1%) compared to Ugandan children (6.7%). During the six-month follow-up, 31.1% of Kenyan and 14.3% of Ugandan children experienced at least one episode of malaria infection. Malaria incidence rate per child-year of follow-up was 0.6 in Kenya and 0.3 in Uganda.

Table 5.1. Baseline characteristics of study participants

| | Kenya, n=1309 | Uganda, n=1374 |
|---|-----------------|-----------------|
| Mean age in years (range) | 2.3 (0.0, 7.1) | 2.3 (1.0, 5.1) |
| Sex, n/total (%) | | |
| Males | 668/1309 (51.0) | 696/1374 (51.7) |
| Females | 641/1309 (49.0) | 678/1374 (49.3) |
| Malaria parasitaemia, n/total (%) | 261/1296 (20.1) | 92/1371 (6.7) |
| Inflammation, n/total (%) ¹ | 334/1264 (26.4) | 316/1337 (23.6) |
| Iron deficiency, n/total (%) ² | | |
| Low ferritin | 457/1237 (36.9) | 438/1267 (34.6) |
| TSAT<10% | 637/1215 (52.4) | n/a |
| Anaemia, n/total (%) ³ | 526/765 (68.8) | 533/1312 (40.6) |
| Iron deficiency anaemia, n/total (%) ⁴ | 172/729 (23.6) | 213/1209 (17.6) |
| Sickle cell trait, n (%) | 157/1057 (14.9) | 224/1355 (16.5) |
| Ferritin, n (gmean±sd) in µg/L | 1237 (20.8±3.0) | 1267 (20.8±2.9) |
| Hepcidin, n (gmean±sd) in µg/L | 1202 (5.6±3.6) | 1333 (6.8±3.3) |
| sTfR, n (gmean±sd) in mg/L | 1296 (17.8±1.5) | 1343 (6.7±2.0) |
| Haemoglobin, n (gmean±sd) in g/dL | 765 (10.1±1.2) | 1312 (11.0±1.1) |
| Transferrin saturation, n (gmean±sd) in % | 1215 (9.3±2.2) | n/a |

gmean, geometric mean; sd, standard deviation; sTfR, soluble transferrin receptors; TSAT, transferrin saturation

¹Inflammation was defined as C-reactive protein > 5mg/L

²Iron deficiency was defined using two definitions: 1) Low ferritin defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation in children < 5 years or < 15µg/L in children ≥ 5 years; and 2) Transferrin saturation < 10% (available in 1215 Kenyan children only and not available (n/a) in Uganda).

³Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years. The range of haemoglobin was 5.1-14.7 in Kenya and 5.4-18.5 in Uganda while interquartile range (IQR) was 9.4-11.3 in Kenya and 10.3-12.1 in Uganda. Only 33 (1.6%) had severe anaemia (Hb<7g/dL in under 5 years or <8g/dL in over 5 years old).

⁴Iron deficiency anaemia was defined as low ferritin and anaemia.

Malaria incidence rate per child-year of follow-up was 0.6 in Kenya and 0.3 in Uganda.

5.4.2 Higher ferritin concentrations and TSAT are positively associated with malaria infection

Concentrations of ferritin and TSAT, but not other iron markers, were higher in children that subsequently developed a malaria episode (Figure 5.2). Similarly, a unit increase in log ferritin was associated with an increased incidence rate ratio for malaria overall (IRR = 1.3; 95% CI: 1.2, 1.4; $P < 0.001$) and in each cohort individually (Figure 5.3). A unit increase in log TSAT was also associated with a 20% increased risk of malaria in Kenyan children (IRR = 1.2; 95%CI: 1.05, 1.4); $P = 0.009$; Figure 5.3). However, hepcidin, sTfR and haemoglobin concentrations were not associated with subsequent risk of malaria (Figure 5.3).

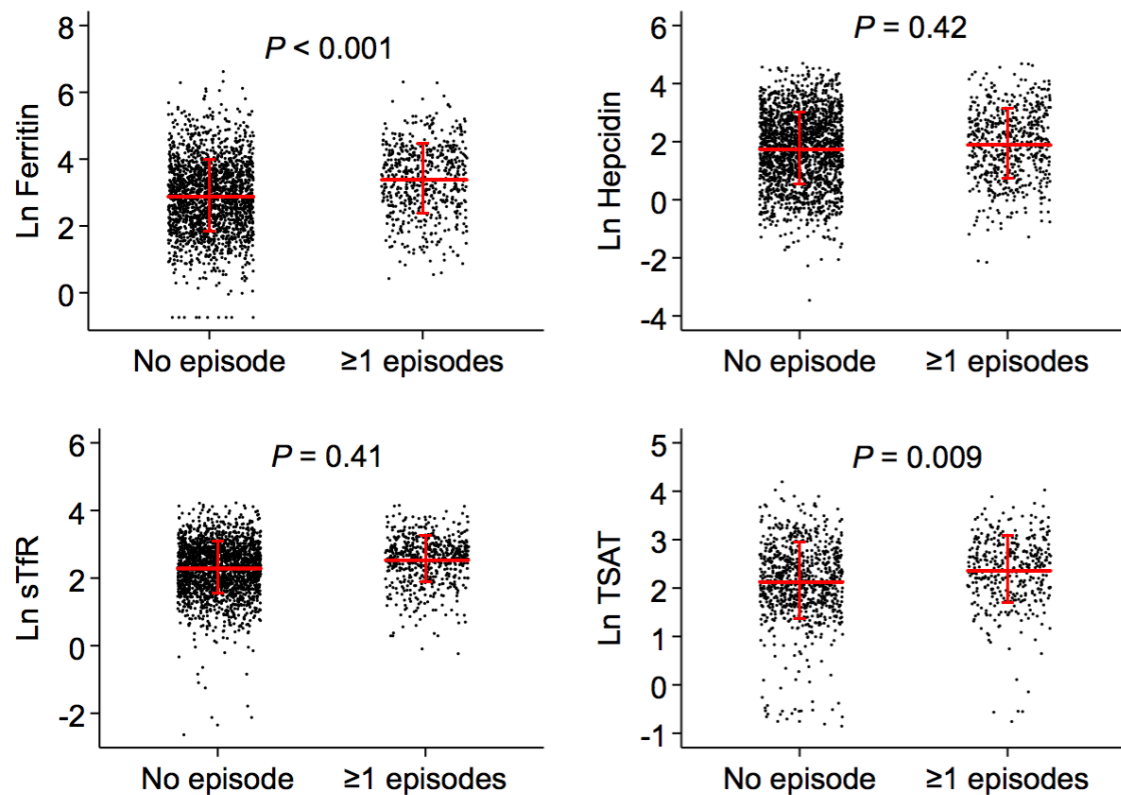


Figure 5.2. Scatter plots of iron biomarkers stratified by no subsequent malaria episodes or one or more malaria episodes.

Horizontal red line indicates mean while vertical line indicates standard deviation. P value was derived from Poisson regression model. sTfR, soluble transferrin receptor; TSAT, transferrin saturation.

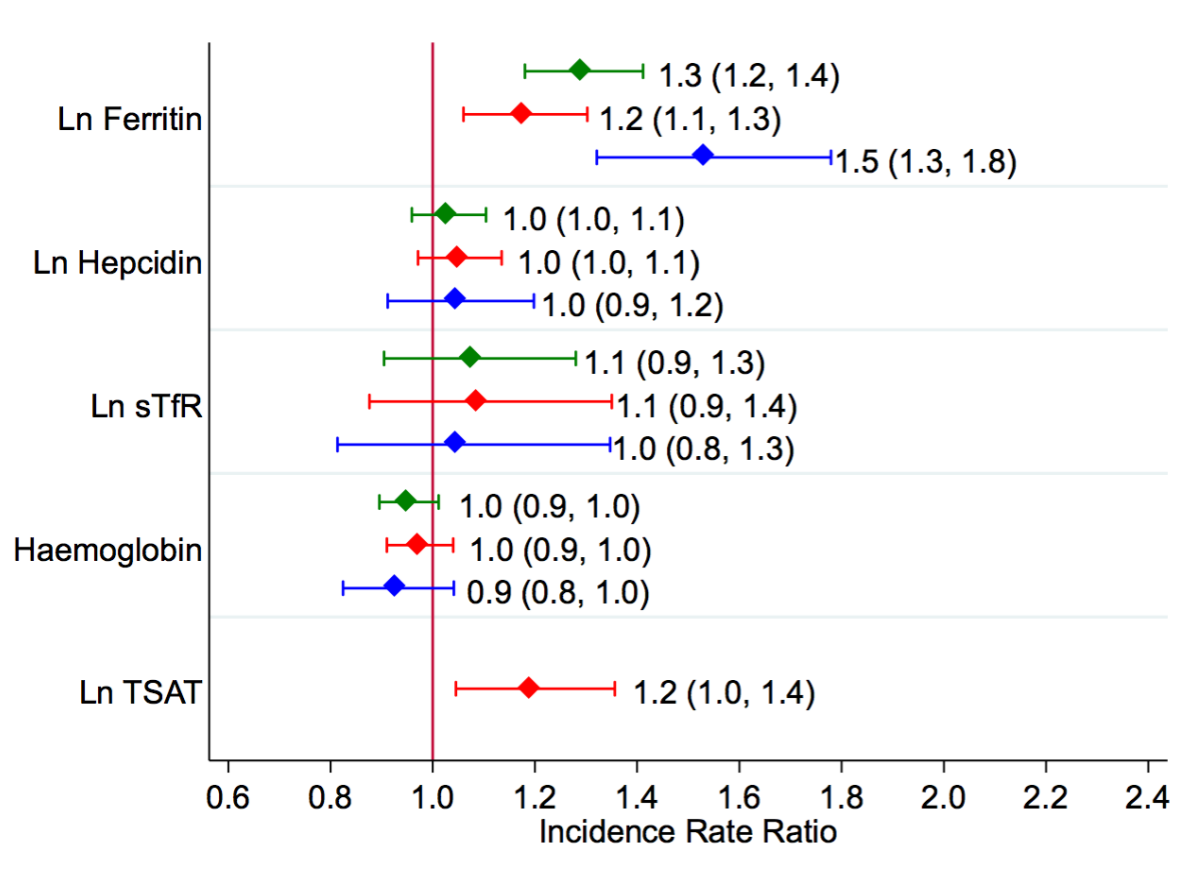


Figure 5.3. Adjusted incidence rate ratios for the effect of iron biomarkers on subsequent malaria episodes.

Green indicates overall, red Kenya and blue Uganda. Labels indicate incidence rate ratio and 95% confidence intervals. Poisson regression models were adjusted for age, sex, parasitaemia, inflammation, length of follow-up and study site. sTfR, soluble transferrin receptor; TSAT, transferrin saturation.

5.4.3 Iron deficiency defined using ferritin or TSAT is associated with protection against malaria risk

Iron deficiency defined by low ferritin concentrations, and iron deficiency anaemia, were associated with 33% and 28% reduction in the incidence of malaria infection (IRR = 0.67; 95% CI: 0.55, 0.82; $P < 0.001$ and IRR = 0.72; 95% CI: 0.56, 0.93; $P = 0.006$, respectively). These findings were consistent for the individual cohorts (Table 5.2). Likewise, iron deficiency defined by low TSAT was associated with a 21% reduction in the risk of malaria in Kenyan children (IRR = 0.79; 95% CI: 0.65, 0.96; $P = 0.016$). However, anaemia itself was not significantly associated with variation in malaria risk (Table 5.2).

Table 5.2. Incidence of malaria by iron status and anaemia

| | Kilifi, Kenya | | | | | Entebbe, Uganda | | | | | Pooled | | | | |
|--------------------------------|---------------|-----------------|-----------|-------------------|-------|-----------------|-----------------|-----------|-------------------|--------|--------|-----------------|-----------|-------------------|--------|
| | No. | No. of episodes | Incidence | IRR (95% CI) | P | No. | No. of episodes | Incidence | IRR (95% CI) | P | No. | No. of episodes | Incidence | IRR (95% CI) | P |
| No ID (iron replete) | 780 | 286 | 0.8 | 1 | | 829 | 140 | 0.3 | 1 | | 1609 | 426 | 0.6 | 1 | |
| ID (low ferritin) ¹ | 457 | 93 | 0.4 | 0.76 (0.59, 0.96) | 0.018 | 438 | 45 | 0.2 | 0.52 (0.36, 0.76) | <0.001 | 895 | 138 | 0.3 | 0.67 (0.55, 0.82) | <0.001 |
| No ID (TSAT ≥10%) | 578 | 224 | 0.8 | 1 | | n/a | n/a | n/a | n/a | n/a | 578 | 224 | 0.8 | 1 | |
| ID (TSAT <10%) ² | 637 | 159 | 0.5 | 0.79 (0.65, 0.96) | 0.016 | n/a | n/a | n/a | n/a | n/a | 637 | 159 | 0.5 | 0.79 (0.65, 0.96) | 0.016 |
| No anaemia | 239 | 97 | 0.8 | 1 | | 779 | 93 | 0.2 | 1 | | 1018 | 190 | 0.4 | 1 | |
| Anaemia ³ | 526 | 219 | 0.9 | 0.99 (0.79, 1.18) | 0.774 | 533 | 93 | 0.4 | 1.21 (0.87, 1.69) | 0.156 | 1059 | 312 | 0.6 | 1.13 (0.93, 1.36) | 0.168 |
| No IDA | 557 | 257 | 1 | 1 | | 996 | 154 | 0.3 | 1 | | 1553 | 411 | 0.5 | 1 | |
| IDA ⁴ | 172 | 47 | 0.6 | 0.84 (0.62, 1.15) | 0.269 | 213 | 23 | 0.2 | 0.47 (0.30, 0.74) | <0.001 | 385 | 70 | 0.4 | 0.72 (0.56, 0.93) | 0.006 |

ID, iron deficiency; IDA, iron deficiency anaemia; TSAT, transferrin saturation

¹Iron deficiency (low ferritin) was defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation (CRP > 5mg/L) in children < 5 years or < 15µg/L in children ≥ 5 years otherwise iron replete.

²Transferrin saturation data were available in 1215 Kenyan children. Not available (n/a) for Uganda.

³Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years.

⁴Iron deficiency anaemia was defined as low ferritin and anaemia.

Poisson regression models were adjusted for age, sex, parasitaemia, inflammation, length of follow-up, and study site. Maximum length of follow-up was 6 months. IRR=Incidence rate ratio. Incidence defined as number of malaria episodes per child-year of follow-up. The number of episodes ranged from 0-5 in Kenya and 0-6 in Uganda. 101 Kenyan and 45 Ugandan children had multiple episodes.

In Cox proportional hazards models, iron deficiency defined by low ferritin or by low TSAT, and iron deficiency anaemia were associated with 40%, 20% and 30% reduced risk of malaria respectively (Figure 5.4A-C) for the six months of follow-up compared to iron replete children. However, anaemia was not associated with malaria risk (Figure 5.4D and Table 5.3).

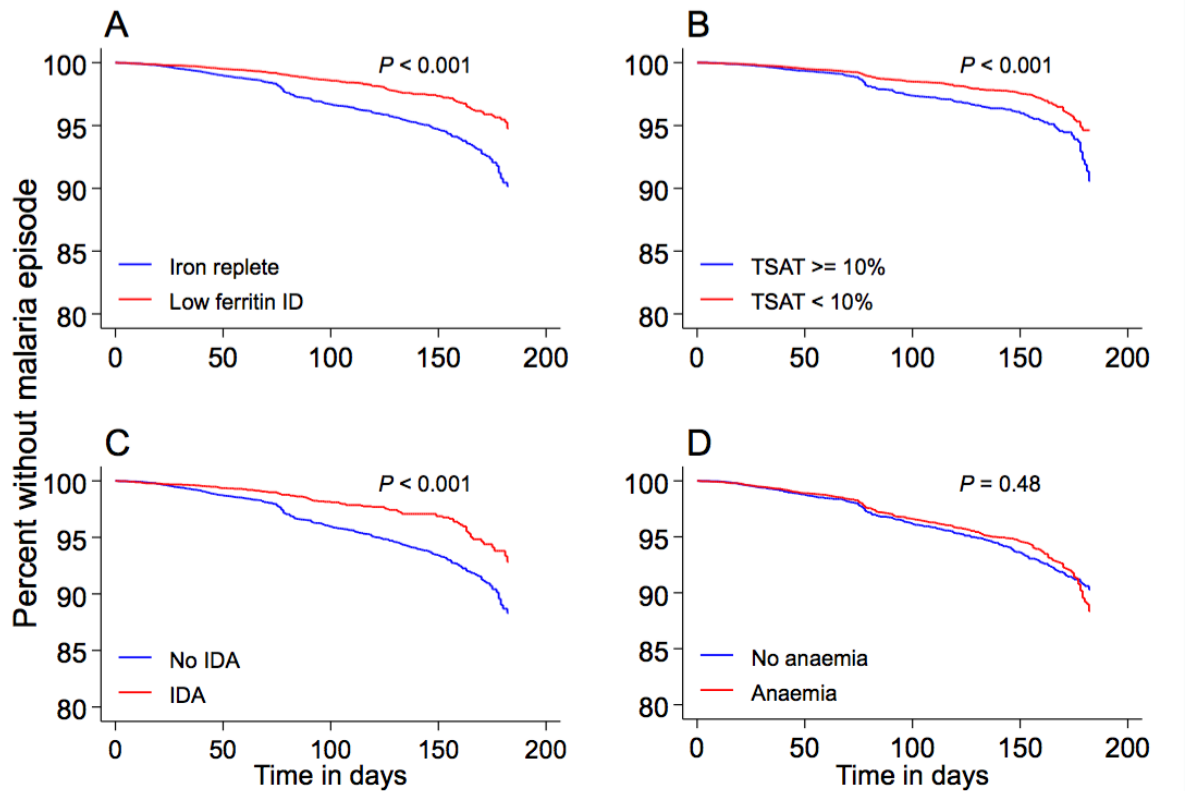


Figure 5.4. Kaplan-Meier curves of time to first malaria episode.

Categorised by A) iron deficiency (ID) defined by low ferritin, B) iron deficiency defined by transferrin saturation (TSAT $< 10\%$), C) iron deficiency anaemia (IDA), and D) anaemia. P values were derived from log-rank tests for equality of survivor functions.

Table 5.3. Cox proportional hazards regression models for predicting malaria risk

| | Unadjusted HR (95% CI) | P value | Adjusted ¹ HR (95% CI) | P value |
|--------------------------------------|---------------------------|---------|--------------------------------------|---------|
| ID (low ferritin) ² | 0.5 (0.4, 0.6) | <0.001 | 0.6 (0.5, 0.7) | <0.001 |
| ID (TSAT<10%) | 0.6 (0.5, 0.7) | <0.001 | 0.8 (0.6, 0.9) | 0.008 |
| Anaemia ³ | 0.9 (0.8, 1.1) | 0.488 | 1.2 (0.9, 1.4) | 0.085 |
| Iron deficiency anaemia ⁴ | 0.5 (0.4, 0.6) | <0.001 | 0.7 (0.6, 0.9) | 0.003 |

ID, iron deficiency; HR, Hazard ratio; TSAT, transferrin saturation

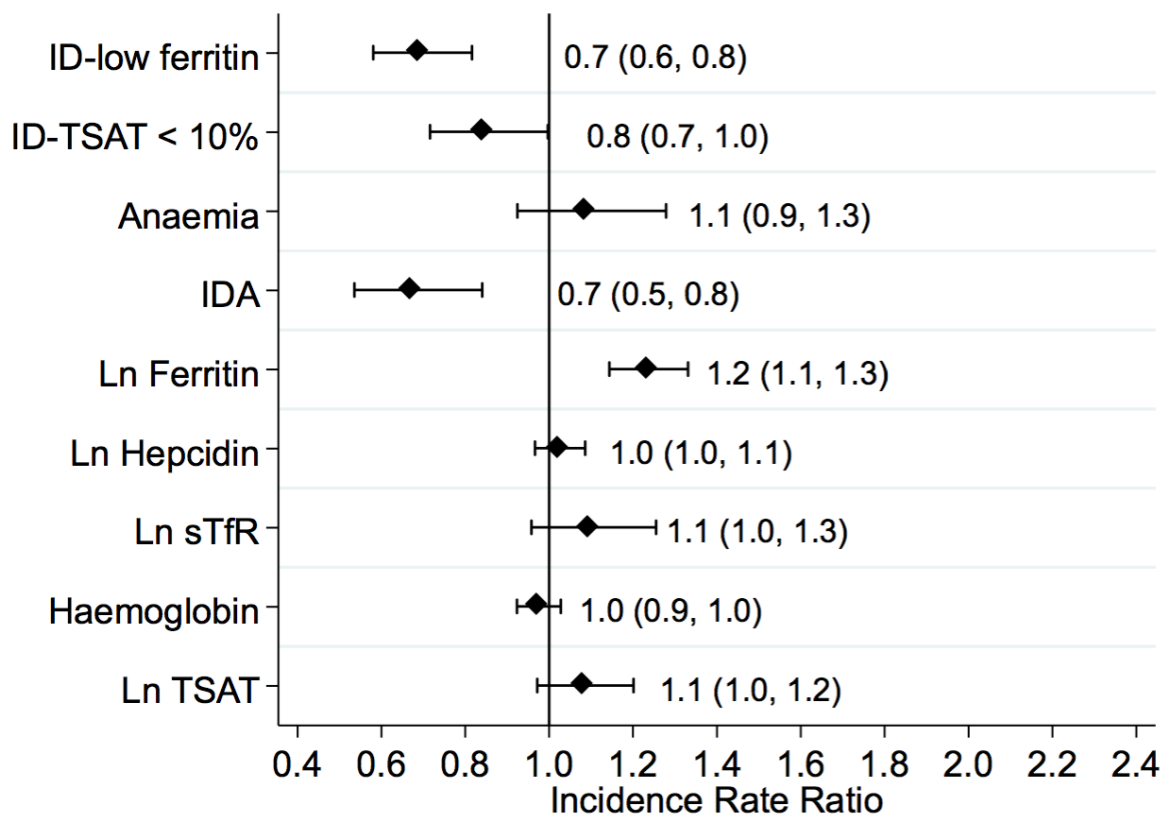
¹Models were adjusted for age, sex, cohort and inflammation.

²ID (low ferritin) was defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation (CRP > 5mg/L) in children < 5 years or < 15µg/L in children ≥ 5 years.

³Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years.

⁴Iron deficiency anaemia was defined as low ferritin and anaemia.

In secondary analyses, I observed similar results after extending the follow-up period to one year (Figure 5.5). Figure 5.5 shows that iron deficiency defined either using low ferritin or TSAT was associated with a reduced risk of malaria even after extending the follow up period to one year.

**Figure 5.5. Effect of iron status on malaria risk over a one-year period.**

Labels indicate incidence rate ratio and 95% confidence intervals. ID is iron deficiency; IDA, iron deficiency anaemia; sTfR, soluble transferrin receptor; TSAT, transferrin saturation. ID-low ferritin was defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation (CRP > 5mg/L) in children < 5 years or < 15µg/L in children ≥ 5 years otherwise, iron replete. Anaemia was defined as hemoglobin < 11g/dL in children aged 0 to 4

years or haemoglobin < 11.5 g/dL in children above 4 years. Iron deficiency anaemia was defined as low ferritin and anaemia. Poisson regression models were adjusted for age, sex, parasitaemia, inflammation, length of follow-up, and study site.

Further secondary analyses included determining the influence of inflammation, malaria parasitaemia and age at baseline on risk of malaria. Exclusion of individuals with inflammation or malaria parasitaemia at baseline did not change the results obtained when all groups were included (Figure 5.6). Similarly, I observed a trend towards protection in all age groups (Figure 5.7).

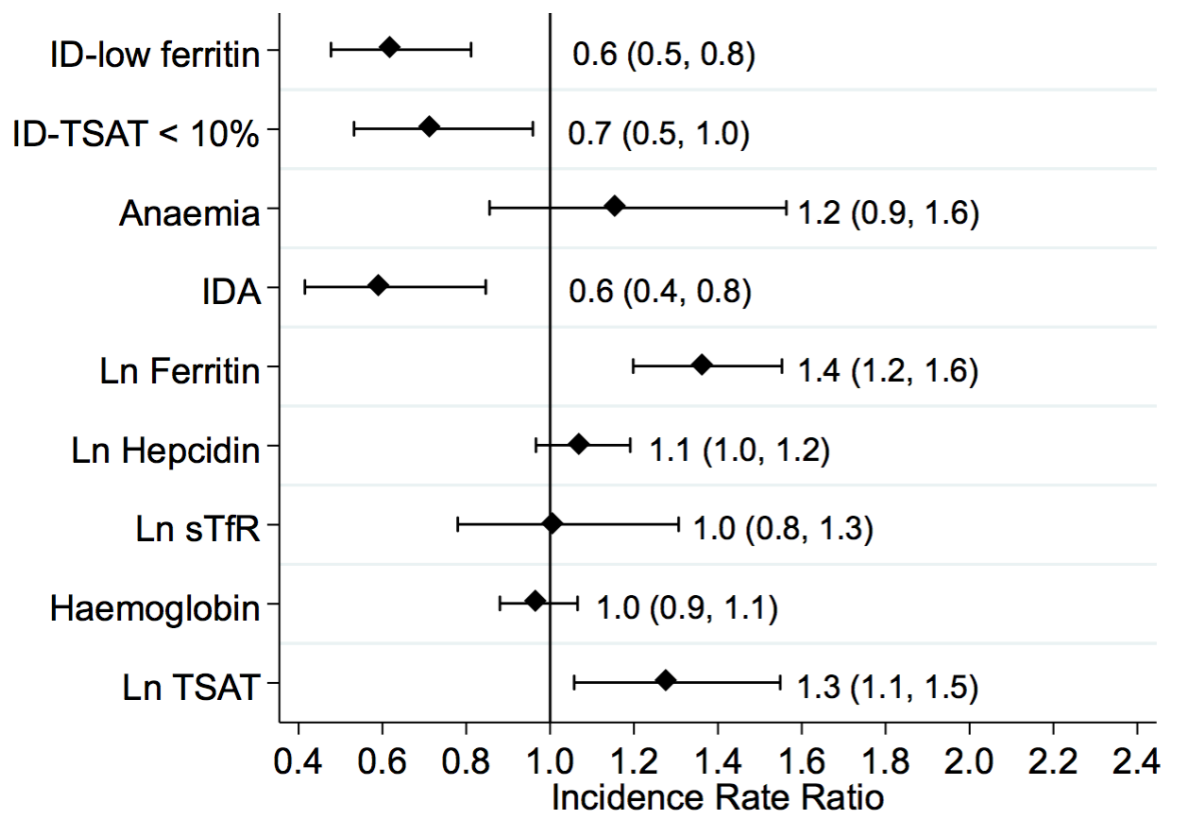


Figure 5.6. Effect of iron status on malaria risk over a six-month period excluding children with inflammation and parasitaemia at baseline.

Labels indicate incidence rate ratio and 95% confidence intervals. Inflammation was defined as C-reactive protein > 5mg/L. ID is iron deficiency; IDA, iron deficiency anaemia; sTfR, soluble transferrin receptor; TSAT, transferrin saturation. ID-low ferritin iron deficiency was defined as plasma ferritin < 12 µg/L in children < 5 years or < 15µg/L in children ≥ 5 years otherwise, iron replete. Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years. Iron deficiency anaemia was defined as low ferritin and anaemia. Poisson regression models were adjusted for age, sex, length of follow-up, and study site. Numbers are 660 in Kenya and 969 in Uganda.

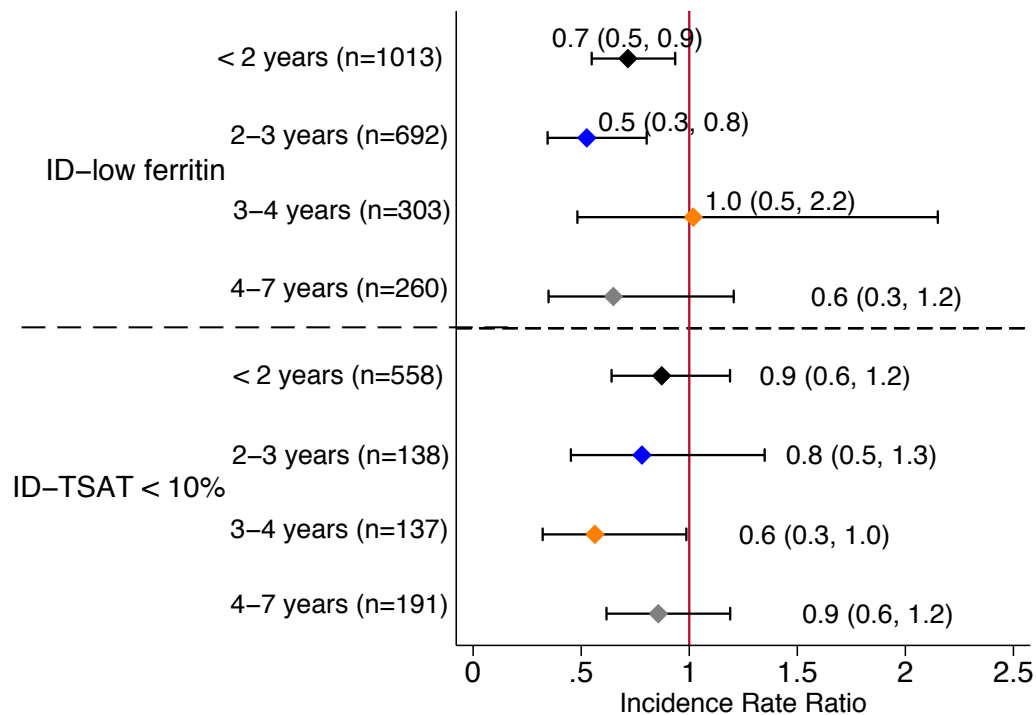


Figure 5.7. Effect of iron deficiency on malaria risk by age group.

Labels indicate incidence rate ratio and 95% confidence intervals. TSAT, transferrin saturation. ID-low ferritin was defined as plasma ferritin < 12 μ g/L or < 30 μ g/L in the presence of inflammation (CRP > 5mg/L) in children < 5 years or < 15 μ g/L in children \geq 5 years.

Since sickle cell trait protects from the risk of malaria (Taylor, Parobek and Fairhurst, 2012), and may also influence iron status (as shown in Chapter Four), I additionally adjusted for its effect in Poisson regression models adjusted for age, sex, parasitaemia, inflammation, length of follow-up, and study site during the six month maximum follow-up period. I observed that additional adjustment for sickle cell trait did not change the results (Figure 5.8).

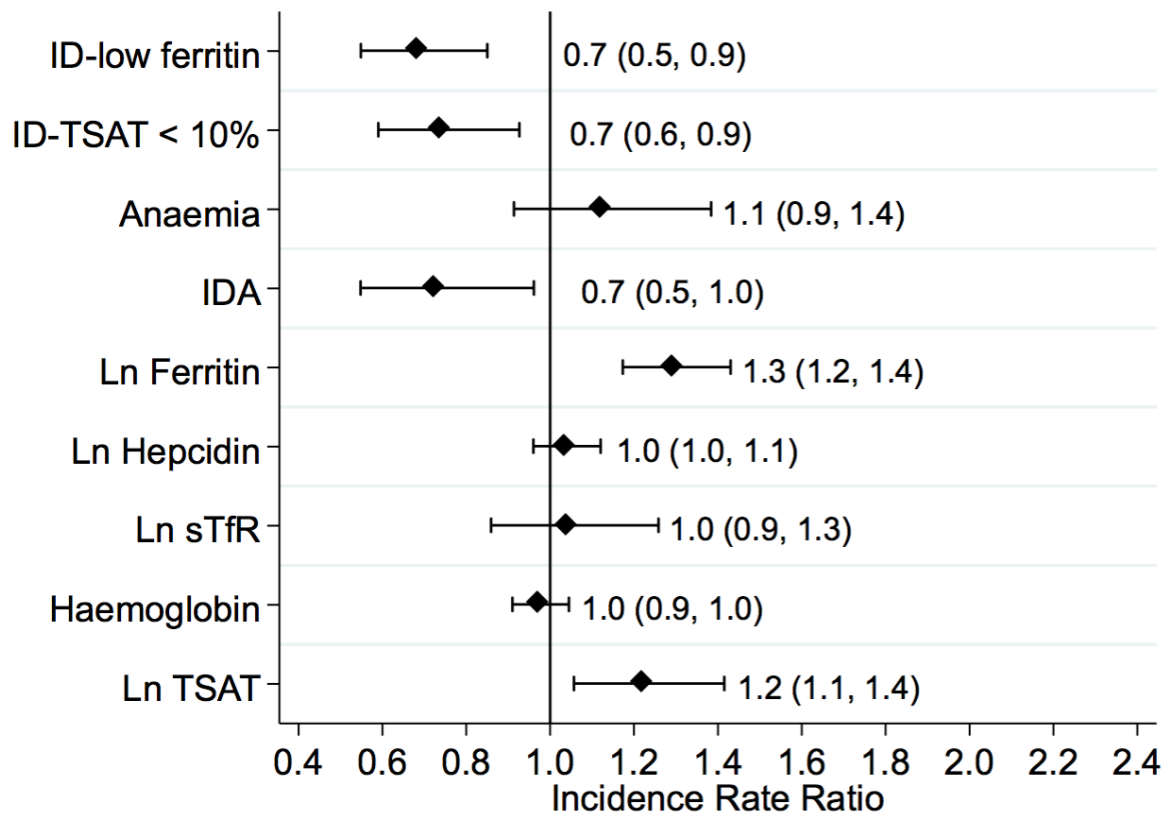


Figure 5.8. Effect of iron status on malaria risk including adjustment for sickle cell trait.

Labels indicate incidence rate ratio and 95% confidence intervals. ID is iron deficiency; IDA, iron deficiency anaemia; sTfR, soluble transferrin receptor; TSAT, transferrin saturation. ID-low ferritin was defined as plasma ferritin < 12µg/L or < 30µg/L in the presence of inflammation (CRP > 5mg/L) in children < 5 years or < 15µg/L in children ≥ 5 years otherwise, iron replete. Anaemia was defined as haemoglobin < 11g/dL in children aged 0 to 4 years or haemoglobin < 11.5 g/dL in children above 4 years. Iron deficiency anaemia was defined as low ferritin and anaemia. Poisson regression models were adjusted for age, sex, parasitaemia, inflammation, length of follow-up, sickle cell trait and study site.

I finally reviewed all previous observational studies (Table 5.4) and performed meta-analysis of the effect of iron deficiency on malaria risk (Figure 5.9). All the studies reported that iron deficiency, using a ferritin-based definition, protects against malaria infection, despite differences in study site, length of follow-up, and definition of iron deficiency (Table 5.4). The overall estimate indicates that iron deficiency is associated with a 34% lower risk of malaria infection (Figure 5.9).

Table 5.4. A summary of observational studies examining the relationship between iron deficiency and malaria risk

| Study | Country | Age (months) | Sample size | Length of follow-up | ID definition | Effect (95% CI) |
|----------------------------------|------------------|--------------|-------------|---------------------|---|--|
| (Nyakeriga <i>et al.</i> , 2004) | Kenya | 8 to 96 | 240 | 6 months | Ferritin<12 μ g/L plus TSAT<10% | 0.70 (0.51, 0.99) ¹ |
| (Jonker <i>et al.</i> , 2012) | Malawi | 6 to 60 | 727 | 12 months | Ferritin<30 μ g/L | 0.49 (0.33, 0.73) |
| (Gwamaka <i>et al.</i> , 2012) | Tanzania | 0 to 36 | 785 | 3 years | Ferritin<30 μ g/L if CRP<8.2mg/L or <70 μ g/L if CRP>8.2mg/L | 0.77 (0.66, 0.89) |
| (Barfffour <i>et al.</i> , 2017) | Zambia | 48 to 72 | 745 | 6 months | Ferritin<12 μ g/L in children<5 years or <15 μ g/L in children \geq 5 years | 0.63 (0.35, 1.11) ² |
| Current study | Kenya and Uganda | 0 to 84 | 2504 | 6 months | Ferritin<12 μ g/L or <30 μ g/L if CRP>5mg/L in children<5 years or <15 μ g/L in children \geq 5 years TSAT<10% | 0.67 (0.56, 0.81) 0.71 (0.54, 0.95) |

¹Children with inflammation and malaria parasitaemia at baseline were excluded.

²Indicates incidence rate ratio of parasitaemia recalculated as inverse of what was reported (1.60 (0.90, 2.86)). Ferritin concentrations were corrected for baseline inflammation (measured using alpha 1-acid glycoprotein) and malaria.

CRP, C-reactive protein; ID, Iron deficiency; TSAT, transferrin saturation.

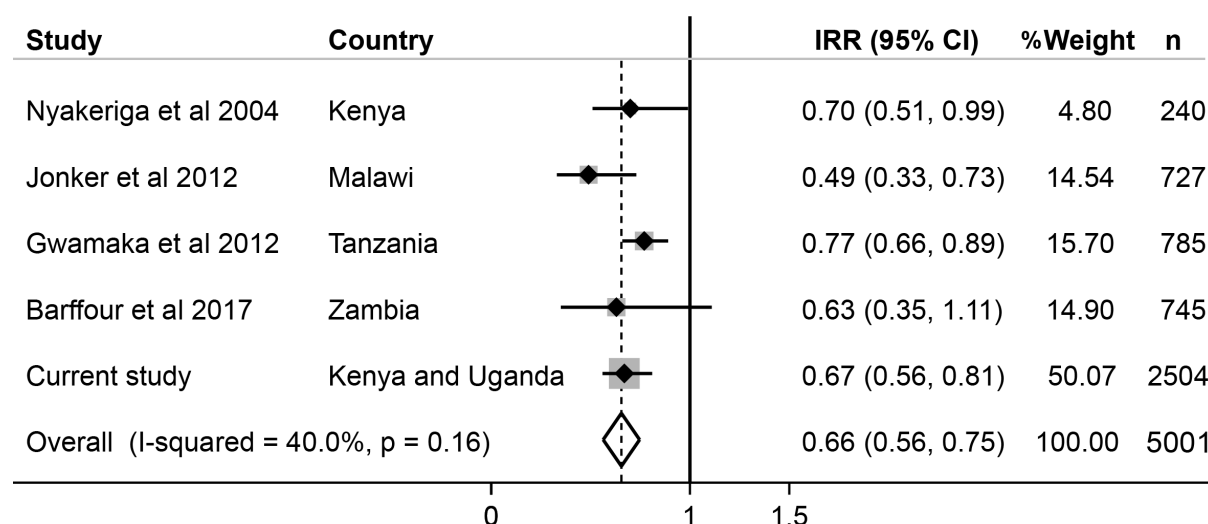


Figure 5.9. Meta-analysis of observational studies examining the relationship between iron deficiency and malaria risk.

Study-specific estimates and their relative contribution (percentage weight and sample size) to overall estimates are shown. Definitions of iron deficiency varied by study as shown in Table 5.4.

5.5 Discussion

In this chapter, I report observational analyses of the influence of iron status on subsequent risk of malaria in 2683 Kenyan and Ugandan children. I found that iron deficiency, defined using either low ferritin or TSAT levels, and iron deficiency anaemia were associated with a lower risk of subsequent malaria episodes. However, anaemia (or haemoglobin concentrations), and concentrations of hepcidin and sTfR were not significantly associated with variation in risk of malaria.

In agreement with these findings, previous observational studies (Table 5.4) have reported that iron deficiency based on low ferritin concentrations confers protection against malaria infection in African children (Nyakeriga *et al.*, 2004; Gwamaka *et al.*, 2012; Jonker *et al.*, 2012; Barffour *et al.*, 2017; Moya-Alvarez *et al.*, 2017). In these studies, the protection ranged from 23% to 45%. These estimates are similar to my finding of a 30% reduction in malaria risk in

iron deficient children aged 0 - 7 years) although length of follow-up differed from that of (Jonker *et al.*, 2012) and (Gwamaka *et al.*, 2012). In a meta-analysis of the current analyses and previous studies, I observed that iron deficiency based on low ferritin concentrations is associated with a 34% reduction in malaria risk.

I further found that iron deficiency defined by TSAT was associated with a 20% reduction in the subsequent risk of malaria and TSAT was positively associated with malaria incidence. Similarly, (Nyakeriga *et al.*, 2004) reported a 30% reduction in clinical malaria among iron deficient Kenyan children using a combination of low TSAT and low ferritin. In support of these findings, *P. falciparum* has been demonstrated to obtain iron from transferrin using *in vitro* parasite culture (Pollack and Fleming, 1984). Furthermore, (Clark *et al.*, 2013) demonstrated that parasitised red blood cells utilise serum iron. These *in vitro* studies suggest that increasing bioavailable transferrin-bound iron through iron supplementation may predispose an individual to increased risk of malaria. This may explain my finding that higher TSAT, which I calculated using serum iron and transferrin, may increase malaria risk in children.

There are a number of possible mechanisms through which iron deficiency may protect from malaria risk. Iron deficient human erythrocytes are poorly infected by *P. falciparum* compared to those that are iron replete and this protective effect is reversed by iron supplementation in *in vitro* parasite cultures (Clark *et al.*, 2014). In mouse models, (Matsuzaki-moriya *et al.*, 2011) showed that during iron deficiency, macrophages cleared parasitised erythrocytes more efficiently suggesting that either erythrocytes produced under iron deficient conditions are easily phagocytosed by macrophages or that macrophage function may be enhanced during iron deficiency. Moreover, iron deficiency has also been shown to up-regulate nitric oxide

which has anti-parasitic properties against *Plasmodium* parasites (Fritsche *et al.*, 2001). Another possible explanation is that during iron deficiency, zinc is incorporated in place of iron during haem synthesis leading to formation of zinc protoporphyrin that in turn is thought to inhibit formation of haemozoin, the parasite survival pigment, in a manner similar to quinolines (Iyer *et al.*, 2003). These possible mechanisms may explain how iron deficiency might protect against risk of malaria infection.

Anaemia (or haemoglobin concentration) was not associated with subsequent malaria risk in my analyses. Similar observations have been reported by two previous studies. Haemoglobin concentrations were not associated with subsequent malaria infection in a longitudinal study in Papua New Guinean infants aged three months and followed-up for one year (Lombardo *et al.*, 2017). A similar observation was made by (Ghosh *et al.*, 1995) in Indian children. My findings and those from these previous studies show that anaemic children have a non-significant increase in malaria risk rather than protection from malaria. Contrary to these findings, (Goheen *et al.*, 2016) reported that anaemia was associated with decreased *in vitro* growth rate of *P. falciparum*. However, it is possible that *in vitro* parasite growth rate might not mimic direct malaria susceptibility for children. Moreover, haemoglobin has low sensitivity and specificity in determining body iron status due to the overlap of values in iron deficient and replete individuals (Cook and Smith, 1976) and the multiple overlapping causes of anaemia in African children (Foote *et al.*, 2013). It also remains unclear whether the malaria parasite utilises haem iron in haemoglobin or has other sources and mechanisms of iron acquisition. In mice, knock-out of *FPN* gene caused accumulation of iron within red blood cells and increased parasite density (Zhang *et al.*, 2018). There are suggestions that the parasite may utilise storage iron since bioavailable iron content increases in parasitised red blood cells as the parasite develops from ring stage to schizont (Clark *et al.*, 2013).

I hypothesised that raised hepcidin concentrations may reduce malaria risk through sequestering iron within macrophages and enterocytes (Nemeth, Tuttle, *et al.*, 2004), thereby starving liver-stage *Plasmodium* (Portugal *et al.*, 2011). Since the parasite requires iron for growth, it has been suggested that withholding iron from hepatocytes inhibits the development of malaria (Portugal *et al.*, 2011). Furthermore, high cord blood hepcidin has been associated with decreased risk of clinical malaria although not parasitaemia or severe malaria in Tanzanian infants (Brickley *et al.*, 2016). However, in agreement with my analyses, an independent cohort of Kenyan children (Atkinson *et al.*, 2015), found no association between hepcidin concentrations and subsequent clinical malaria episodes. Differences in age or environmental factors may account for the different findings. Moreover, a possible protective role of hepcidin may be countered by the high prevalence of iron deficiency that I observed in this population. Iron deficiency is normally associated with decreased hepcidin (Atkinson *et al.*, 2014) and is protective against malaria risk. In fact, I observed a trend towards a positive association between hepcidin concentrations and malaria risk. Hepcidin may increase malaria risk through degradation of erythrocytic ferroportin, which leads to accumulation of iron within erythrocytes and thus may favour intraerythrocytic proliferation of malaria parasites (Zhang *et al.*, 2018). Moreover, hepcidin may increase spread of the malaria parasites to other red blood cells since accumulation of iron leads to increased oxidative stress and haemolysis (Zhang *et al.*, 2018).

I found that a child's erythropoietic drive (as indicated by sTfR) did not influence their subsequent risk of malaria infection. The expression of sTfR increases with both iron deficiency and expanded erythropoiesis (with the latter being more influential) (Beguin, 2003), factors that might have opposing effects on malaria risk. For example, increased erythropoiesis may increase malaria risk since *Plasmodium* parasites preferentially infect young red blood

cells (Pasvol, Weatherall and Wilson, 1980), whereas iron deficiency may be protective. These opposing effects could explain why sTfR concentrations were not associated with malaria risk in my analyses. Reverse causality is also possible since malaria itself causes increased sTfR concentrations (Verhoef *et al.*, 2001; Beguin, 2003) although the effect did not change after exclusion of individuals with malaria episodes at baseline.

There were limitations and strengths in my analyses. A major challenge in these analyses is that iron biomarkers are themselves influenced by infections and inflammatory processes which may confound the effect of iron status on malaria infection as shown in Chapter Three. To mitigate the potential confounding effects of infection on iron biomarkers, I excluded children with inflammation or malaria parasitaemia at the time of iron measurement in secondary analyses and observed similar results (Figure 5.6). I additionally, adjusted for other potential confounders such as age, sex, length of follow-up and study site in regression models. Furthermore, the protective effect was consistent across age groups and did not change following additional adjustment for sickle cell trait. My analyses were also limited by a lack of TSAT concentrations in Ugandan children. Strengths of my analyses included its large size (n=2,683 children) across two study populations and that I used multiple iron biomarkers in order to determine their individual effects on malaria risk making it the largest and most definitive observational study to address the question of iron status and risk of malaria infection.

5.6 Conclusion

These findings, in agreement with other studies, suggest that iron deficiency protects children against malaria infection and thus that improving iron status may predispose African children to infection. Interestingly, of the iron biomarkers, only higher concentrations of ferritin and TSAT were predictive of increased rates of subsequent malaria, perhaps reflecting differences

in their relationship to parasite mechanisms of iron acquisition. Although WHO recommends iron supplementation coupled with malaria treatment and prevention strategies in malaria endemic areas (World Health Organization, 2016), these strategies remain difficult to implement. Thus, it is important to establish whether improved iron status increases malaria risk since this would necessitate long-term malaria prevention and treatment programs. However, these findings and that of other studies do not necessarily prove causality since observational studies may be subject to confounding and reverse causation, for example, prior malaria exposure might lead to both iron deficiency and the acquisition of protective immunity against malaria, while malaria itself increases ferritin concentrations. Since iron deficiency prevents children from reaching their developmental potential it is important to establish causality in the iron-malaria relationship. Thus, these data warrant further large-scale studies, including Mendelian randomisation studies, which utilise genetic variants associated with iron status to infer causality or prospective randomised controlled trials.

CHAPTER SIX: FERROPORTIN Q248H IS ASSOCIATED WITH PROTECTION AGAINST IRON DEFICIENCY AND ANAEMIA BUT NOT MALARIA

The material in this chapter forms the basis of a published paper in *Science Advances* entitled “*The ferroportin Q248H mutation protects from anemia, but not malaria or bacteremia*”, in which I was the first author.

6.1 Abstract

Introduction: Ferroportin is the sole known iron exporter in mammals. Recently, ferroportin was shown to export iron from mature red blood cells and deletion of the *FPN* gene resulted in haemolytic anaemia and increased parasite density and deaths in malaria-infected mice. The *FPN* Q248H mutation renders ferroportin partially resistant to hepcidin-induced degradation and was associated with protection from malaria in a study of limited size. I therefore aimed to determine whether the *FPN* Q248H mutation influences iron status and malaria infection in African children.

Methods: I used data for 3374 children aged from 3 months to 7 years from community-based cohorts in Kenya, Uganda, Burkina Faso, South Africa, and The Gambia, and 11,982 children from severe malaria (case-control) studies in The Gambia, Malawi, Kenya, and Ghana. I fitted regression models to determine the effect of the *FPN* Q248H mutation on iron status, anaemia, and malaria infection.

Results: Overall, the prevalence of the *FPN* Q248H mutation (homozygotes and heterozygotes) was 10.5% but varied by study site ranging from 8.6% in Uganda to 16.5% in South Africa. The *FPN* Q248H mutation was associated with a trend towards improved iron status with a 23% reduced odds of iron deficiency (OR = 0.77; 95% CI 0.60, 0.99; $P = 0.046$), and a 25% reduced odds of anaemia (OR = 0.75; 0.57, 0.98; $P = 0.037$). There was little evidence of

protection against either uncomplicated malaria (OR = 1.06 (0.68, 1.64; $P = 0.79$) or severe malaria (OR = 0.91; 95% CI 0.81, 1.01; $P = 0.08$).

Conclusion: The ferroportin Q248H variant was associated with modest protection against iron deficiency and anaemia but there was little evidence of protection against malaria. Moreover, the mutation does not seem to have been selected due to malaria exposure as it occurred in highest frequency in South Africa, a cohort with no malaria historically.

6.2 Introduction

Malaria parasites and other infectious pathogens require iron to grow and multiply. The human host has therefore evolved mechanisms to withhold iron from pathogens using iron-binding and chaperone transport proteins (Barber and Elde, 2014). Ferroportin, the only known iron exporter is highly expressed in duodenal enterocytes, hepatocytes, and splenic macrophages to allow iron absorption, storage and recycling (Donovan *et al.*, 2000). The iron hormone, hepcidin, regulates ferroportin in response to iron status, erythropoiesis, and inflammation. Hepcidin occludes and degrades ferroportin thereby reducing cellular iron export (Nemeth, Tuttle, *et al.*, 2004; Aschemeyer *et al.*, 2018). A recent study showed that ferroportin is also abundantly expressed on mature red blood cells and that knock-out of the *FPN* gene resulted in the accumulation of excess intracellular iron, haemolytic anaemia and increased parasite density and death in malaria-infected mice (Zhang *et al.*, 2018).

FPN Q248H, a mutation in the *FPN* gene in humans, may increase cellular iron export by rendering ferroportin partially resistant to hepcidin degradation (Nekhai *et al.*, 2013). However, some studies have shown that the Q248H impairs cellular iron export (McGregor *et al.*, 2005) or has no functional effect (Drakesmith *et al.*, 2005; Schimanski *et al.*, 2005). Furthermore, previous studies with limited sample sizes, have found conflicting associations between Q248H

and haemoglobin concentrations (Beutler *et al.*, 2003; Gordeuk *et al.*, 2003; McNamara, Gordeuk and MacPhail, 2005; Masaisa *et al.*, 2012; Zhang *et al.*, 2018) or with iron deficiency (Beutler *et al.*, 2003; Gordeuk *et al.*, 2003; Kasvosve *et al.*, 2005, 2010, 2015; Rivers *et al.*, 2007; Nekhai *et al.*, 2013; Cikomola *et al.*, 2017).

The Q248H mutation occurs mainly in African populations and it has been hypothesised that the mutation has been positively selected due to protection from malaria (Zhang *et al.*, 2018) (reviewed in Chapter One). By increasing cellular iron export, *FPN* Q248H may deprive *P. falciparum*, an intracellular pathogen, of iron, limiting its survival. In a recent study, (Zhang *et al.*, 2018) reported that Q248H mutation is associated with lower *P. falciparum* parasite density compared to the wild type allele in a small sample size of Zambian children and primiparous Ghanaian women. Moreover, (Zhang *et al.*, 2018) included only children with uncomplicated malaria without matched controls and therefore could not assess malaria risk. In this Chapter, I investigated whether the Q248H mutation is associated with protection from iron deficiency, anaemia, and malaria infection using large-scale datasets of African children.

6.3. Methods

6.3.1 Study populations

To test the effect of *FPN* Q248H mutation on iron status, anaemia and uncomplicated malaria, I used data from community-based cohorts in Kilifi, Kenya; Entebbe, Uganda; Soweto, South Africa; Banfora, Burkina Faso; and West Kiang, The Gambia. These studies are described in Chapter Two Section 2.1. In this Chapter, I also analysed haptoglobin concentrations that were measured in The Gambia only using the Tina Quant Haptoglobin Kit, Roche Diagnostics, Cobas Bio centrifugal analyzer. I further tested the effect of the *FPN* Q248H mutation on the risk of severe malaria using case-control data of severe malaria in children from Kenya,

Malawi, The Gambia, and Ghana recruited as part of MalariaGEN (<https://www.malariagen.net/data/genome-wide-study-resistance-severe-malaria-eleven-populations>). Severe malaria was diagnosed as *P. falciparum* parasites in the blood film plus clinical features of severe malaria including haemoglobin <5g/dL / haematocrit level of <15% (for severe malarial anaemia) or Blantyre coma score of <3 (for cerebral malaria) (Malaria Genomic Epidemiology Network, 2014). With the help of Dr Gavin Band, a member of the MalariaGEN consortium and based at the University of Oxford, I retrieved the Q248H mutation from the MalariaGEN genome-wide association study datasets and analysed the effect of the mutation on severe malaria in the Kenyan dataset. Analyses for Malawi, The Gambia, and Ghana severe malaria datasets were performed by Dr Gavin Band and are reproduced in this thesis with permission as published in (Muriuki, Mentzer, Band, *et al.*, 2019).

6.3.2 Statistical analysis

In all community-based cohorts, I conducted all statistical analyses using STATA 13.0 (StataCorp., College Station, TX). I normalised individual iron markers by ln-transformation. I compared proportions between groups by Chi-square or Fisher's exact test where appropriate, and continuous variables by Student's *t*-test or Mann Whitney U test. I fitted linear and logistic regression models adjusting for age, sex and cohort. I performed meta-analyses using the 'metan' package in STATA. Analyses of MalariaGEN severe malaria data were undertaken using logistic regression in R and multinomial regression in SNPTTEST (version 2.5) respectively. For the malaria case-control analysis undertaken for trios in Ghana, a transmission disequilibrium test was undertaken using the 'trio' package in R. In all analyses, I assumed a dominant model, comparing Q248H heterozygote and homozygote carriers to non-carriers since there were very few homozygotes for any meaningful analyses. A p-value of <0.05 was considered significant and all p-values reflect a two-tailed test. I also conducted a literature

review and meta-analysis of all published papers reporting association between Q248H and iron status, anaemia, or inflammation.

6.4 Results

6.4.1 Characteristics of study participants

Table 6.1 shows the characteristics of the community-based populations included in this study. I tested for associations between the *FPN* Q248H mutation and anaemia and measures of iron status among 3374 children aged from 3 months to 7 years from community-based cohorts in Uganda, The Gambia, Burkina Faso, Kenya, and South Africa. Overall, the Q248H mutation was observed among 10.5% of children ($n = 355$ Q248H carriers, 342 heterozygotes and 13 homozygotes combined assuming a dominant mode of action). In the MalariaGEN severe malaria case-control datasets, there were a total of 11,982 children, 5489 hospitalised patients with severe malaria ($n = 658$ Q248H carriers) and 6493 matched controls ($n = 1519$ Q248H carriers) from The Gambia, Malawi, Kenya, and Ghana.

Where appropriate, I used Chi-square, Fisher's exact or Student's t tests to explore the relationship between iron status / anaemia and Q248H mutation (Table 6.1). I observed a trend towards improved iron status in children carrying the Q248H mutation compared to wild type across the study sites (Table 6.1).

Table 6.1. Characteristics of participants by study cohort and *FPN* Q248H mutation

| | Uganda | | | The Gambia | | | South Africa | | | Burkina Faso | | | Kenya | | |
|-------------------------------------|------------------|----------------|------|-----------------|---------------|------|------------------|---------------|------|-----------------|---------------|------|-----------------|---------------|-------|
| | Q248H (n=117) | WT (n=1248) | P | Q248H (n=71) | WT (n=675) | P | Q248H (n=102) | WT (n=518) | P | Q248H (n=34) | WT (n=313) | P | Q248H (n=31) | WT (n=265) | P |
| Median age in years (IQR) | 2.0 (2.0-3.0) | 2.0 (2.0-3.0) | 0.90 | 3.8 (2.9-4.5) | 3.8 (2.9-4.9) | 0.56 | 1.0 (1.0-1.0) | 1.0 (1.0-1.0) | 0.35 | 1.9 (1.6-2.1) | 1.9 (1.7-2.2) | 0.32 | 4.2 (2.1-6.1) | 4.1 (2.3-6.0) | 0.87 |
| Females, n (%) | 49 (41.9) | 622 (49.8) | 0.10 | 32 (45.1) | 315 (46.7) | 0.80 | 46 (45.1) | 253 (48.8) | 0.49 | 18 (52.9) | 154 (49.2) | 0.68 | 13 (41.9) | 127 (47.9) | 0.53 |
| Iron deficiency, n (%) ¹ | 38 (34.2) | 397 (34.6) | 0.94 | 11 (15.7) | 149 (22.1) | 0.22 | 34 (33.3) | 222 (42.9) | 0.07 | 11 (36.7) | 104 (35.5) | 0.90 | 4 (33.3) | 61 (57.0) | 0.12 |
| Anaemia, n (%) ² | 36 (33.6) | 493 (41.2) | 0.13 | 32 (46.4) | 377 (56.3) | 0.12 | n/a | n/a | NA | 24 (77.4) | 263 (88.0) | 0.10 | 19 (63.3) | 172 (65.4) | 0.82 |
| IDA, n (%) ³ | 15 (14.7) | 197 (17.9) | 0.42 | 8 (11.8) | 109 (16.3) | 0.33 | n/a | n/a | NA | 8 (29.6) | 88 (31.3) | 0.86 | 4 (36.4) | 41 (39.1) | 0.57 |
| Haemoglobin, g/dL | 11.2 (1.1) | 11.0 (1.1) | 0.26 | 10.8 (1.1) | 10.5 (1.1) | 0.12 | n/a | n/a | NA | 9.8 (1.1) | 9.5 (1.1) | 0.10 | 10.6 (1.1) | 10.2 (1.2) | 0.34 |
| MCHC, g/dL | 33.0 (1.0) | 32.9 (1.0) | 0.72 | n/a | n/a | NA | n/a | n/a | NA | n/a | n/a | NA | 33.5 (1.1) | 32.4 (1.1) | 0.001 |
| MCV, fL | 70.6 (1.1) | 71.1 (1.1) | 0.51 | 75.3 (1.1) | 75.6 (1.1) | 0.72 | n/a | n/a | NA | n/a | n/a | NA | 76.5 (1.1) | 73.5 (1.1) | 0.07 |
| Haptoglobin, μ mol/L | n/a | n/a | NA | 71.1 (3.5) | 69.6 (3.0) | 0.04 | n/a | n/a | NA | n/a | n/a | NA | n/a | n/a | NA |
| Ferritin, μ g/L | 20.9 (2.4) | 19.6 (2.9) | 0.54 | 29.4 (2.5) | 24.8 (2.4) | 0.13 | 17.4 (2.5) | 14.5 (2.6) | 0.08 | 22.1 (3.0) | 22.0 (2.3) | 0.98 | 19.2 (3.8) | 12.2 (3.4) | 0.23 |
| Hepcidin, μ g/L | 7.2 (3.2) | 6.7 (3.3) | 0.60 | 12.2 (4.0) | 11.2 (4.7) | 0.68 | 9.4 (2.9) | 7.4 (3.6) | 0.08 | 6.0 (4.3) | 5.3 (4.2) | 0.62 | 3.7 (4.6) | 3.8 (3.9) | 0.93 |
| sTfR, mg/L | 6.6 (1.9) | 6.8 (2.0) | 0.80 | 3.4 (1.5) | 3.4 (1.4) | 0.99 | 10.4 (1.5) | 10.8 (1.5) | 0.36 | 16.3 (1.8) | 17.9 (1.7) | 0.36 | 16.7 (1.7) | 14.9 (1.5) | 0.37 |
| Iron, μ mol/L | n/a | n/a | NA | 9.0 (1.7) | 8.6 (1.6) | 0.46 | n/a | n/a | NA | 6.4 (1.6) | 6.0 (1.8) | 0.55 | 3.5 (2.3) | 2.7 (3.1) | 0.41 |
| Transferrin, g/L | 2.7 (1.3) | 2.7 (1.3) | 0.90 | n/a | n/a | NA | 2.5 (1.3) | 2.7 (1.3) | 0.06 | 2.7 (1.2) | 2.7 (1.3) | 0.81 | 2.9 (1.2) | 3.0 (1.2) | 0.37 |
| TSAT, % | n/a | n/a | NA | 13.3 (1.8) | 12.8 (1.7) | 0.58 | n/a | n/a | NA | 9.5 (1.8) | 8.9 (2.0) | 0.59 | 3.7 (2.6) | 3.4 (3.4) | 0.81 |
| ZPP, μ mol/mol haem | n/a | n/a | NA | 97.4 (1.8) | 114.6 (1.8) | 0.03 | n/a | n/a | NA | n/a | n/a | NA | n/a | n/a | NA |
| CRP/ACT, mg/L/g/L | 1.1 (4.6) | 1.4 (5.2) | 0.11 | 0.5 (1.3) | 0.4 (1.3) | 0.12 | 0.8 (5.4) | 0.9 (4.9) | 0.86 | 2.2 (5.3) | 2.9 (6.2) | 0.41 | 1.2 (4.5) | 1.1 (4.8) | 0.86 |

Values are geometric means (\pm SD) for the various biomarkers. Proportions were compared between groups by Chi-square or Fisher's exact test where appropriate, and continuous variables by Student's t-test or Mann Whitney U test (Age).

Q248H includes both heterozygotes and homozygotes.

¹Iron deficiency defined as plasma ferritin < 12 μ g/L or < 30 μ g/L in the presence of inflammation (C-reactive protein >5mg/L α 1-antichymotrypsin >0.6g/L in The Gambia) in children < 5 years or < 15 μ g/L in children \geq 5 years

²Anaemia defined as hemoglobin < 11g/dL

³IDA, iron deficiency anaemia defined as iron deficiency and anaemia

ACT, α 1-antichymotrypsin; CRP, C-reactive protein; IQR, interquartile range; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; sTfR, soluble transferrin receptor; TSAT, transferrin saturation; ZPP, zinc protoporphyrin; n/a, not available; NA, not applicable; WT, wild type.

6.4.2 *FPN* Q248H is associated with modest protection against anaemia

In regression analyses, I first evaluated the effect of the mutation on the risk of anaemia in 2666 children with haemoglobin measurements. In pooled analyses, the prevalence of anaemia (Hb < 11 g/dl) was lower in Q248H children (46.8%) compared to those carrying the wild type (53.7%; OR = 0.75; 0.57, 0.98; $P = 0.037$; Figure 6.1A, Table 6.2). Mean haemoglobin concentrations were 0.22 g/dL higher in Q248H children compared to WT (10.81 g/dl vs. 10.59 g/dl; $P = 0.036$) (Figure 6.1B, Table 6.2).

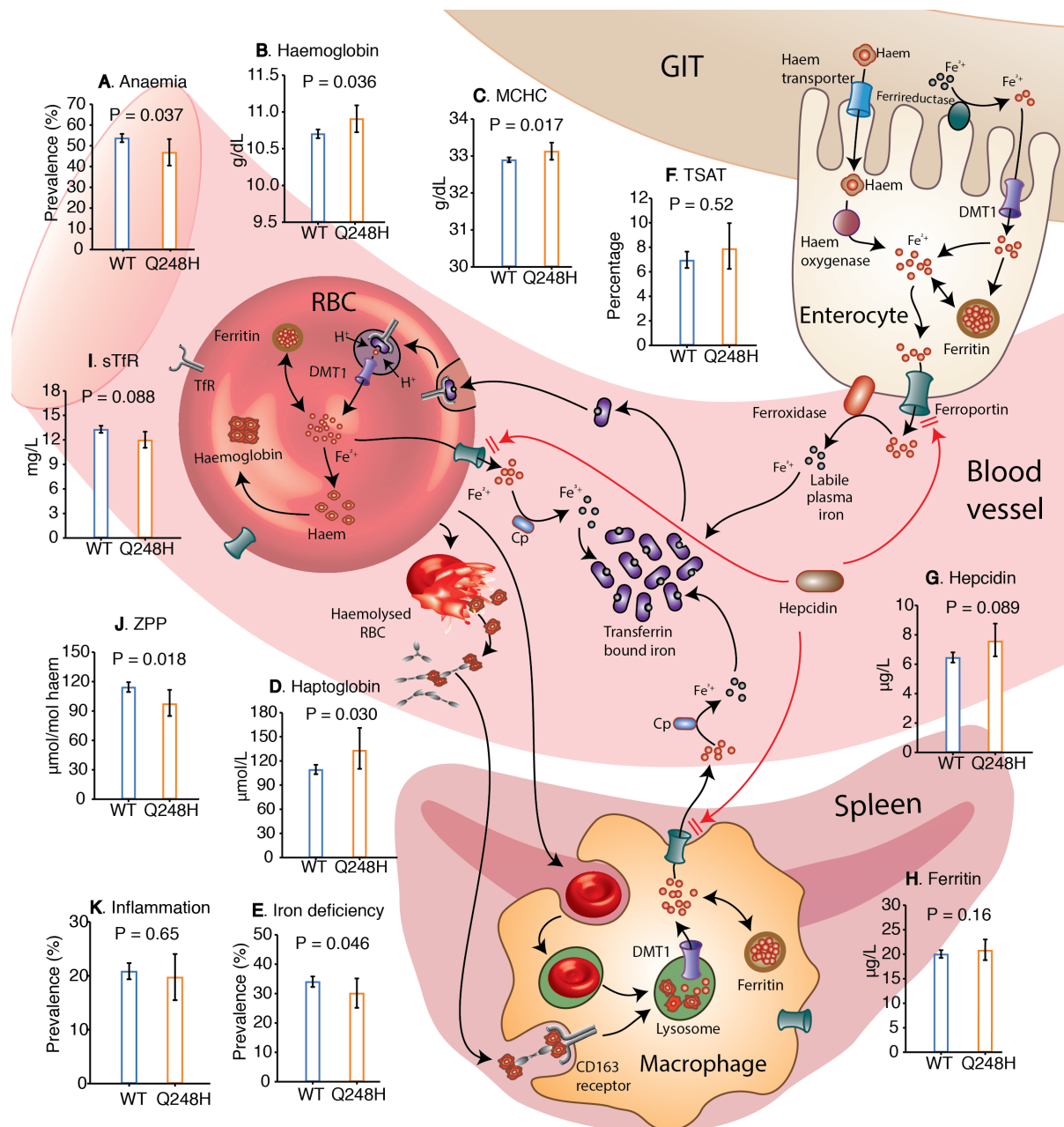


Figure 6.1. Association between *FPN* Q248H mutation and anaemia, red cell haemolysis and iron deficiency (ID).

The Q248H mutation renders ferroportin partially resistant to hepcidin degradation (4) increasing iron export through enterocytes, erythrocytes and macrophages. The effects of Q248H on (A) anaemia, (B) haemoglobin, (C) mean corpuscular haemoglobin concentrations (MCHC), (D) haptoglobin, (E) iron deficiency (ID) (F) transferrin saturation, (G) hepcidin, (H) ferritin, (I) soluble transferrin receptors (sTfR), (J) zinc protoporphyrin (ZPP), and (K) inflammation indicate reduced haemolysis, limited effect on iron status and no association with inflammation. Plotted values are geometric means except anaemia, iron deficiency and inflammation, which are percentages. All P values were two-tailed and derived from regression models adjusted for age, sex and cohort. I drew this diagram and it is reproduced from (Muriuki, Mentzer, Band, et al., 2019).

Table 6.2. Association between *FPN* Q248H mutation and anaemia, haemolysis, and iron deficiency in community-based children

| Category | All, n | Q248H ¹ | | WT | | OR (95% CI) | P value |
|--------------------------------------|--------|--------------------|---------------------|-------|---------------------|----------------------|---------|
| | | Q248H, n | n (%) | WT, n | n (%) | | |
| Anaemia ² | 2666 | 237 | 111 (46.8) | 2429 | 1305 (53.7) | 0.75 (0.57, 0.98) | 0.037 |
| Iron deficiency ³ | 3065 | 325 | 98 (30.2) | 2740 | 933 (34.1) | 0.77 (0.60, 0.99) | 0.046 |
| Iron deficiency anaemia ⁴ | 2363 | 208 | 35 (16.8) | 2155 | 435 (20.2) | 0.77 (0.52, 1.14) | 0.19 |
| Inflammation ⁵ | 3149 | 334 | 66 (19.8) | 2815 | 587 (20.9) | 0.94 (0.70, 1.25) | 0.65 |
| Biomarkers | All, n | Q248H, n | Geometric mean (SD) | WT, n | Geometric mean (SD) | Estimate (95% CI) | P value |
| Haemoglobin, g/dL | 2666 | 237 | 10.81 (1.15) | 2429 | 10.59 (1.16) | 0.21 (0.01, 0.40) | 0.036 |
| MCHC, g/dL | 1537 | 131 | 33.10 (1.04) | 1406 | 32.87 (1.04) | 0.27 (0.05, 0.50) | 0.017 |
| MCV, fL | 2324 | 205 | 72.98 (1.12) | 2119 | 72.76 (1.12) | 0.16 (-0.87, 1.19) | 0.76 |
| Haptoglobin, μ mol/L | 587 | 50 | 133.22 (1.98) | 537 | 109.32 (1.87) | 0.20 (0.02, 0.38) | 0.030 |
| Ferritin, μ g/L | 3065 | 325 | 20.80 (2.53) | 2740 | 20.03 (2.84) | 0.08 (-0.03, 0.20) | 0.16 |
| Hepcidin, μ g/L | 3080 | 319 | 8.31 (3.48) | 2761 | 7.33 (3.87) | 0.14 (-0.02, 0.30) | 0.089 |
| sTfR, mg/L | 3091 | 325 | 11.98 (1.66) | 2766 | 13.30 (1.67) | -0.07 (-0.16, 0.01) | 0.088 |
| Iron, μ mol/L | 459 | 45 | 5.45 (1.91) | 414 | 4.83 (2.35) | 0.14 (-0.10, 0.37) | 0.25 |
| Transferrin, g/L | 2380 | 258 | 2.62 (1.28) | 2122 | 2.70 (1.27) | -0.06 (-0.14, 0.02) | 0.14 |
| TSAT, % | 430 | 40 | 7.89 (2.13) | 390 | 6.95 (2.62) | 0.09 (-0.18, 0.36) | 0.52 |
| ZPP, μ mol/mol haem | 684 | 66 | 97.43 (1.80) | 618 | 114.63 (1.77) | -0.17 (-0.31, -0.03) | 0.018 |
| CRP, mg/L | 2403 | 263 | 1.08 (5.07) | 2140 | 1.35 (5.44) | -0.19 (-0.41, 0.02) | 0.079 |
| ACT, g/L | 737 | 68 | 0.47 (1.34) | 669 | 0.44 (1.30) | 0.05 (-0.01, 0.12) | 0.12 |

¹Heterozygotes and homozygotes

²Anaemia was defined as haemoglobin < 11g/dL.

³Iron deficiency was defined as plasma ferritin < 12 μ g/L or < 30 μ g/L in the presence of inflammation in children < 5 years or < 15 μ g/L in children \geq 5 years.

⁴Iron deficiency anaemia was defined as iron deficiency with anaemia.

⁵Inflammation was defined as C-reactive protein >5mg/L or α 1-antichymotrypsin >0.6g/L in The Gambia.

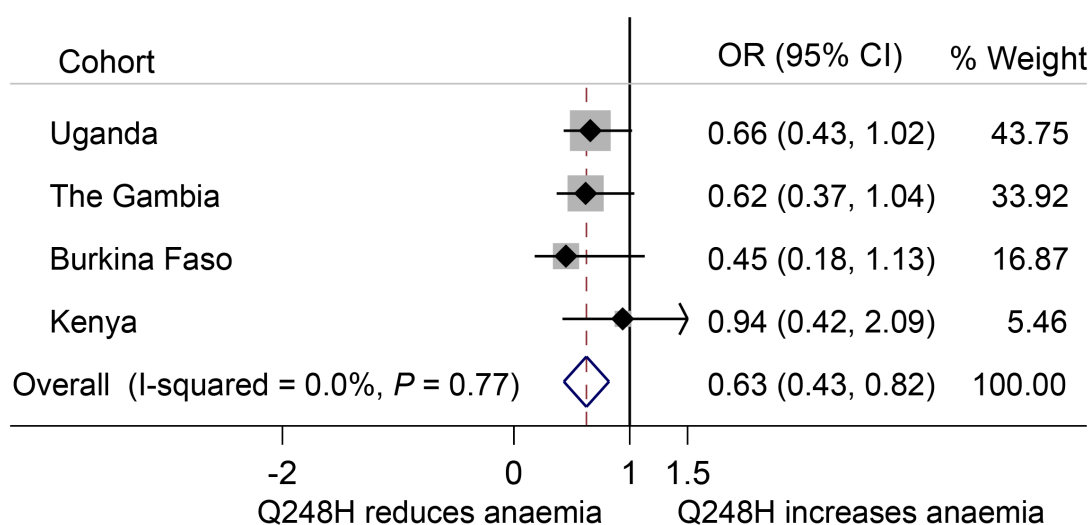
Analyses were adjusted for age, sex and country.

ACT, α 1-antichymotrypsin; CRP, C-reactive protein; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; sTfR, soluble transferrin receptor; TSAT, transferrin saturation; ZPP, zinc protoporphyrin

Since pooling datasets from different populations or assays may be influenced by heterogeneity, I then performed a meta-analysis of cohort-specific associations between the Q248H mutation and anaemia and haemoglobin concentrations. *FPN* Q248H mutation consistently increased haemoglobin concentrations and was protective against anaemia across the study populations (Figures 6.2; $P = 0.0\%$). I also meta-analysed the pooled estimate of the current study and previous published studies (Table 6.3 and Figure 6.3). Overall, the mutation was associated with higher haemoglobin concentrations compared to wild type (Figure 6.3). I

similarly observed higher mean corpuscular haemoglobin concentrations (MCHC) in children carrying Q248H compared to WT ($P = 0.017$, Figure 6.1C), but no evidence of a difference in mean corpuscular volume (MCV) (Table 6.2). I hypothesised that the modestly increased haemoglobin concentrations and MCHC observed in Q248H carriers could be due to reduced haemolysis and / or improved iron status.

Anaemia



Haemoglobin, g/dL

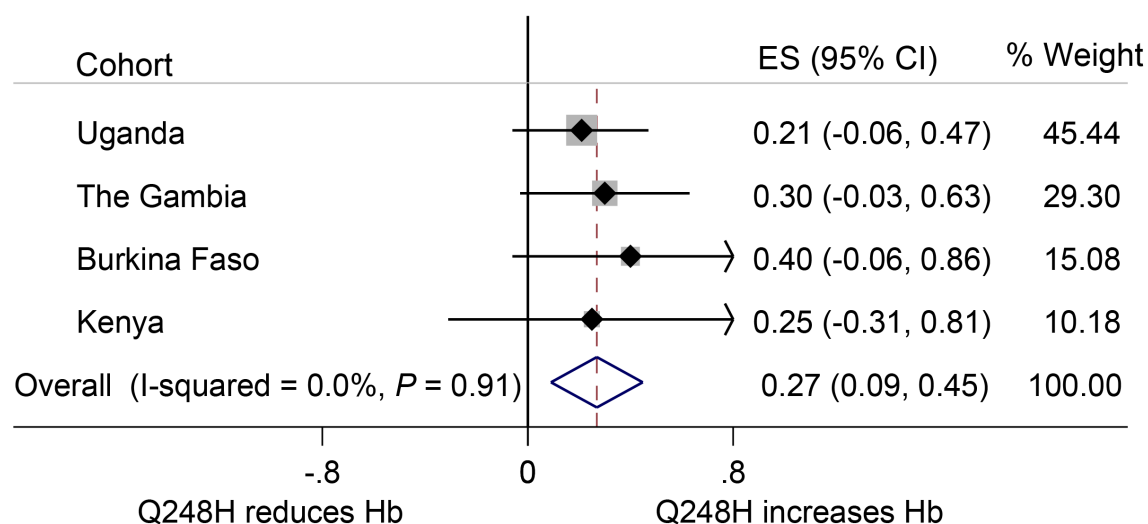


Figure 6.2. Meta-analysis of associations of the *FPN* Q248H mutation with anaemia and haemoglobin across the study populations.

Labels indicate odds ratios or estimates and 95% confidence intervals. Haemoglobin measurements were not available for the South African cohort. Regression models were adjusted for age and sex. OR, Odds Ratio; ES, Effect Size.

Table 6.3. Summary of studies examining the relationship between the *FPN* Q248H mutation and haemoglobin, ferritin, and C-reactive protein

| Study | Country | Age | Sample size | Q248H, n | WT, n | Summary statistic | Q248H | Wild Type (WT) |
|--|----------------------------|----------|-------------|----------|-------|-------------------------|----------------------|----------------------|
| Haemoglobin, g/dl | | | | | | | | |
| (Gordeuk <i>et al.</i> , 2003) ¹ | South Africa and Swaziland | Adults | 51 | 7 | 44 | Mean (\pm SD) | 12.5 (\pm 1.3) | 13.7 (\pm 1.3) |
| (Beutler <i>et al.</i> , 2003) ² , males | USA - African Americans | Adults | 276 | 25 | 251 | Mean (\pm SD) | 14.2 (\pm 1.5) | 14.5 (\pm 1.6) |
| (Beutler <i>et al.</i> , 2003), females | USA - African Americans | Adults | 293 | 40 | 253 | Mean (\pm SD) | 12.5 (\pm 1.3) | 12.6 (\pm 1.6) |
| (McNamara, Gordeuk and MacPhail, 2005) ² | South Africa | Adults | 217 | 53 | 164 | Mean (\pm SD) | 13.6 (\pm 1.5) | 13.5 (\pm 1.3) |
| (Masaisa <i>et al.</i> , 2012) ³ | Rwanda | Women | 200 | 12 | 188 | Mean (\pm SD) | 13.1 (\pm 3.4) | 12.4 (\pm 2.9) |
| (Zhang <i>et al.</i> , 2018) | Zambia | Children | 66 | 13 | 53 | Mean (\pm SD) | 9.2 (\pm 2.5) | 9.5 (\pm 2.2) |
| (Zhang <i>et al.</i> , 2018) | Ghana | Women | 290 | 25 | 265 | Mean (\pm SD) | 11.5 (\pm 1.5) | 11.2 (\pm 1.9) |
| (Kasvosve <i>et al.</i> , 2010) | Zimbabwe | Children | 59 | 13 | 46 | Median (IQR) | 11.2 (10.6, 12.0) | 10.9 (10.3, 11.5) |
| Current study ⁴ | Across Africa | Children | 2666 | 237 | 2429 | Mean (\pm SD) | 10.8 (\pm 1.1) | 10.6 (\pm 1.2) |
| Ferritin, ng/ml or μg/L | | | | | | | | |
| (Gordeuk <i>et al.</i> , 2003) | South Africa and Swaziland | Adults | 51 | 7 | 44 | Geometric mean (95% CI) | 63.0 (40.0, 100.0) | 35.0 (29.0, 42.0) |
| (Beutler <i>et al.</i> , 2003), males | USA - African Americans | Adults | 276 | 25 | 251 | Geometric mean (95% CI) | 141.8 (93.9, 214.4) | 158.4 (141.3, 177.5) |
| (Beutler <i>et al.</i> , 2003), females | USA - African Americans | Adults | 293 | 40 | 253 | Geometric mean (95% CI) | 62.9 (40.5, 97.9) | 51.2 (44.4, 59.0) |
| (Kasvosve <i>et al.</i> , 2005) | Zimbabwe | Children | 195 | 38 | 157 | Geometric mean (95% CI) | 32.0 (27.0, 39.0) | 21.0 (19.0, 23.0) |
| (McNamara, Gordeuk and MacPhail, 2005) | South Africa | Adults | 217 | 53 | 164 | Geometric mean (95% CI) | 163.0 (138.0, 193.0) | 131.0 (119.0, 144.0) |
| (Katchunga <i>et al.</i> , 2013), controls | DRC | Adults | 86 | 7 | 79 | Geometric mean (95% CI) | 105.3 (92.3, 186.7) | 128.3 (65.1, 228.7) |
| (Katchunga <i>et al.</i> , 2013), diabetic | DRC | Adults | 179 | 25 | 154 | Geometric mean (95% CI) | 214.4 (121.2, 309.1) | 183.6 (97.1, 292.6) |
| (Nekhai <i>et al.</i> , 2013) ⁵ | USA | Adults | 78 | 9 | 69 | Geometric mean (95% CI) | 88.0 (29.0, 273.0) | 211.0 (74.0, 602.0) |
| (Kasvosve <i>et al.</i> , 2010) | Zimbabwe | Children | 64 | 14 | 50 | Median (IQR) | 17.0 (11.0, 29.0) | 20.0 (10.0, 33.0) |
| (Masaisa <i>et al.</i> , 2012) | Rwanda | Women | 200 | 12 | 188 | Median (IQR) | 295.0 (179.0, 363.0) | 74.9 (47.2, 118.0) |
| (Kasvosve <i>et al.</i> , 2015) ⁶ , males | Botswana | Adults | 84 | 14 | 70 | Median (IQR) | 127.4 (97.2, 192.5) | 79.7 (50.6, 137.0) |
| (Kasvosve <i>et al.</i> , 2015), females | Botswana | Adults | 74 | 12 | 62 | Median (IQR) | 47.4 (22.2, 83.8) | 38.5 (24.1, 68.1) |
| (Cikomola <i>et al.</i> , 2017) | DRC | Adults | 42 | 15 | 27 | Median (IQR) | 211.0 (142.0, 476.0) | 228.0 (115.0, 540.0) |
| Current study | Across Africa | Children | 3065 | 325 | 2740 | Geometric mean (95% CI) | 20.8 (18.8, 23.0) | 20.0 (19.3, 20.8) |
| C-reactive protein, mg/L | | | | | | | | |
| (Masaisa <i>et al.</i> , 2012) | Rwanda | Women | 200 | 12 | 188 | Mean (\pm SD) | 5.2 (\pm 2.0) | 4.8 (\pm 1.2) |
| (McNamara, Gordeuk and MacPhail, 2005) | South Africa | Adults | 217 | 53 | 164 | Geometric mean (95% CI) | 4.5 (4.0, 5.1) | 2.7 (2.5, 2.9) |
| (Kasvosve <i>et al.</i> , 2005) | Zimbabwe | Children | 195 | 38 | 157 | Median (IQR) | 0.8 (0.3, 2.8) | 0.6 (0.1, 2.5) |
| (Kasvosve <i>et al.</i> , 2010) | Zimbabwe | Children | 64 | 14 | 50 | Median (IQR) | 1.0 (0.3, 2.0) | 0.5 (0.1, 2.0) |
| Current study | Across Africa | Children | 2403 | 263 | 2140 | Geometric mean (95% CI) | 1.1 (0.9, 1.3) | 1.4 (1.3, 1.5) |

¹I used community controls. I calculated standard deviation from the provided standard error.

²Participants had iron overload. I calculated standard deviation from the provided standard error.

³HIV positive women.

⁴The current study included data from Kenya, Uganda, South Africa, Burkina Faso and The Gambia.

⁵The study included patients with sickle cell anaemia and transfused <5 units of blood.

⁶The study analysed data for those with serum ferritin \geq 10ng/mL and \leq 200ng/mL.

CI, confidence interval; DRC, Democratic Republic of Congo; IQR, interquartile range; SD, standard deviation; USA, United States of America.

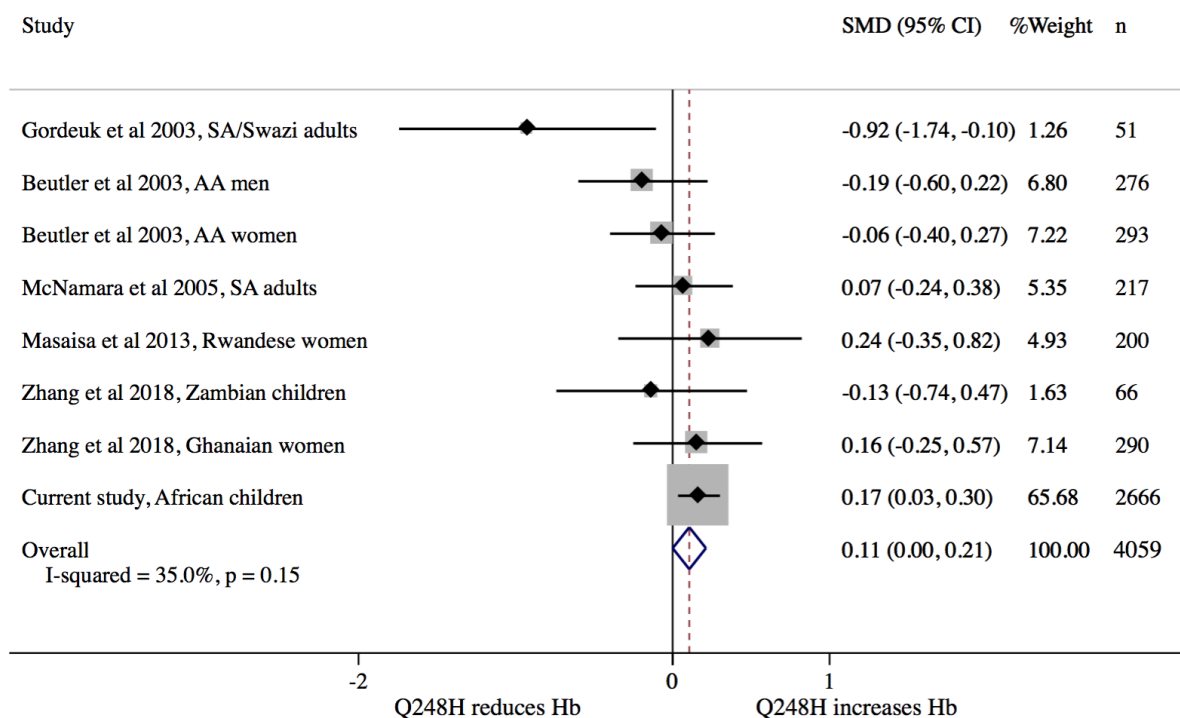


Figure 6.3. A meta-analysis of studies investigating the relationship between the *FPN* Q248H mutation and haemoglobin levels.

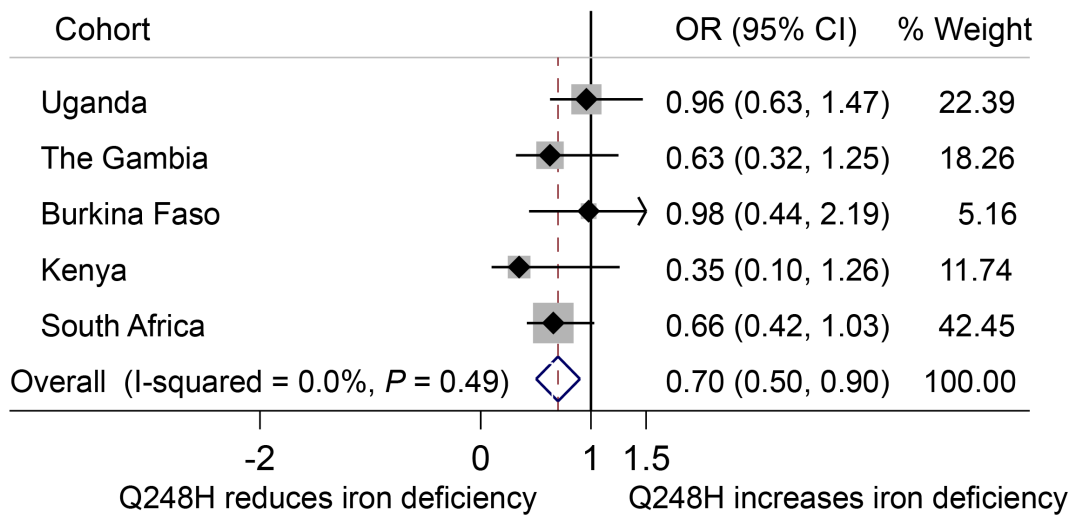
The current study included Ugandan, Gambian, Burkinabe, and Kenyan children. Table 1 gives more details of the studies included in the meta-analysis. The grey boxes indicate sample size. SA, South Africa; AA, African-American; SMD, standardised mean difference.

To test the hypothesis that higher haemoglobin concentrations among Q248H children may be due to reduced haemolysis, I used data for haptoglobin concentrations in 587 Gambian children aged 2 to 6 years. I observed that Q248H carriers ($n = 50$) had higher geometric mean haptoglobin concentrations compared with wild type ($133.22 \mu\text{mol/L}$ vs. $109.32 \mu\text{mol/L}$; $\beta = 0.20$; $0.02, 0.38$; $P = 0.03$) consistent with reduced haemolysis in Q248H children (Figure 6.1D, Table 6.2 above). These findings support the hypothesis that reduced haemolysis may contribute to the higher haemoglobin concentrations observed in Q248H children.

6.4.3 FPN Q248H is associated with protection against iron deficiency

I further hypothesised that higher haemoglobin concentrations could be due to enhanced absorption and recycling of iron among Q248H carriers leading to improved iron status as previously observed in smaller studies (Table 6.3). Among 3065 children, 1031 (33.6%) were iron deficient. In pooled analyses, the Q248H variant ($n = 325$) was associated with a 23% reduction in the odds of iron deficiency ($P = 0.046$; comparing Q248H heterozygotes and homozygotes to non-carriers) and iron deficiency anaemia ($P = 0.19$, Table 6.2). I observed a trend towards improved iron status for individual measures of iron status among Q248H children, however with the exception of ZPP, a sensitive indicator of iron deficiency ($P = 0.018$), none of the associations reached conventional statistical significance (Figure 6.1, Table 6.2). In a meta-analysis of cohort specific associations of Q248H mutation and iron deficiency and ferritin, I observed a 30% protection from iron deficiency and higher ferritin concentrations in individuals carrying the mutation compared to the wild type (Figure 6.4).

Iron deficiency



Ln Ferritin, µg/L

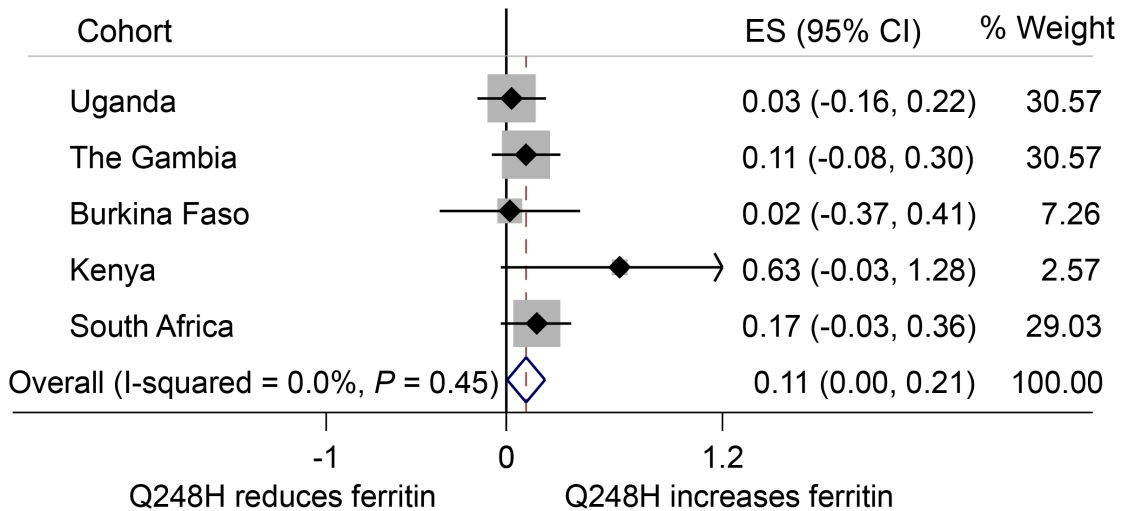


Figure 6.4. Meta-analysis of associations of the *FPN* Q248H mutation with iron status across the study populations.

Labels indicate odds ratios or estimates and 95% confidence intervals. Ferritin was normalised by ln-transformation. Regression models were adjusted for age and sex. OR, Odds Ratio; ES, Effect Size.

Since increased hepcidin expression is a major factor in the alteration of iron stores during inflammation, and Q248H ferroportin may be partially resistant to hepcidin (Nekhai *et al.*, 2013), I stratified analyses by inflammation (Figure 6.5A). The prevalence of inflammation did not differ between Q248H and WT children ($P = 0.65$; Figure 6.1K, Table 6.2). After excluding children with inflammation, I observed a 30% reduction in the odds of iron deficiency and anaemia among Q248H carriers ($n = 2409$, adjusted OR = 0.7; 95% CI 0.5, 0.9; Figure 6.5A). Similarly, the mutation was associated with a significant increase in ferritin concentrations in individuals without inflammation ($\beta = 0.1$; 0.01, 0.2; $P = 0.037$) but not in individuals with inflammation ($\beta = 0.02$; -0.2, 0.3; $P = 0.91$). However, I found no significant interaction between the Q248H mutation and inflammation in predicting iron deficiency and anaemia. I further stratified by low and high hepcidin (using the absorption threshold of 5.5 $\mu\text{g/L}$ (Pasricha *et al.*, 2014)) but found no evidence of an interaction or any effect after stratification (Figure 6.5B). Taken together these findings suggest, in agreement with some previous studies (Table 6.3), that the Q248H mutation is associated with modestly improved iron status in African children.

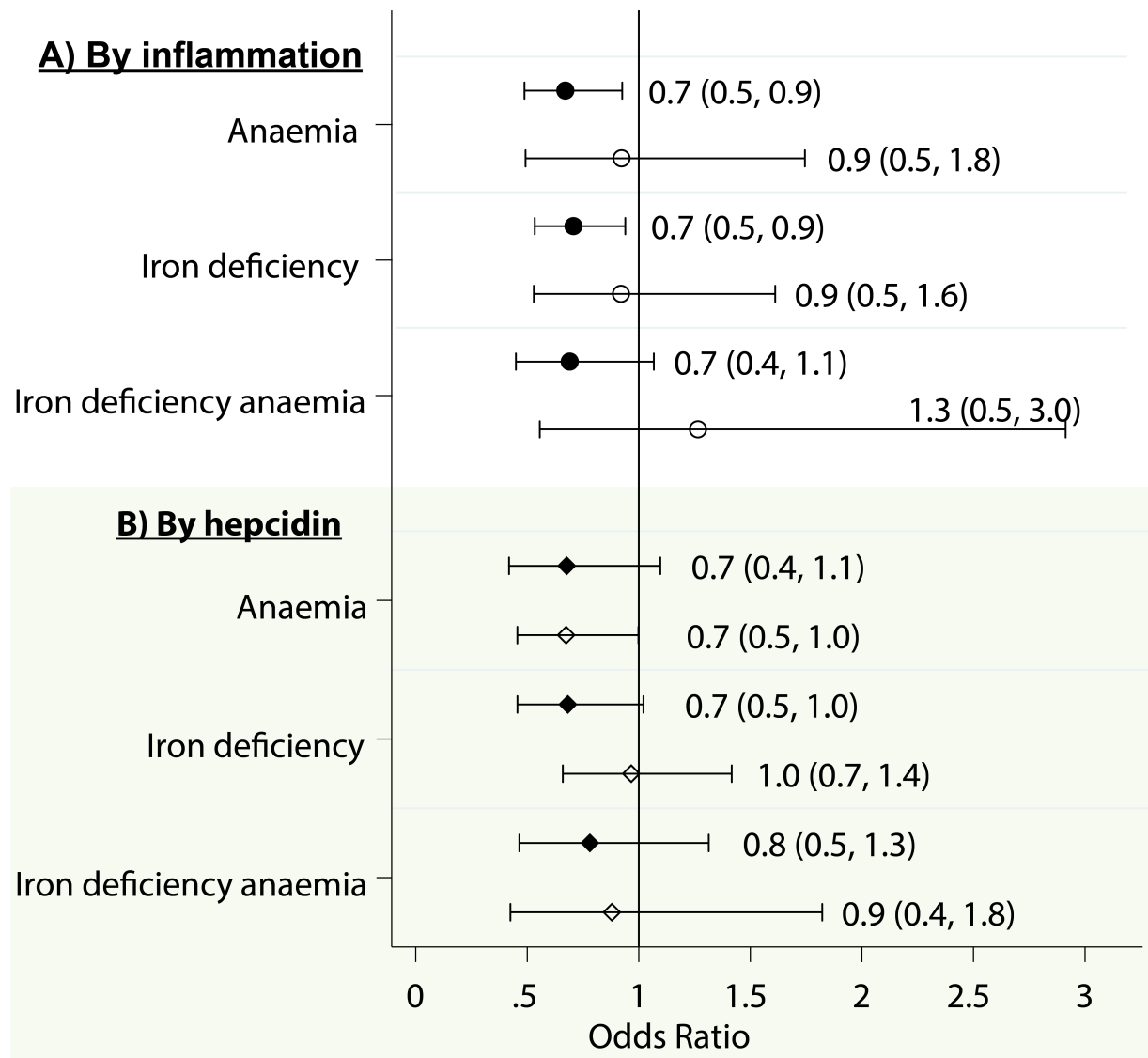


Figure 6.5. Effect of the *FPN* Q248H mutation on anaemia and iron deficiency stratified by inflammation and hepcidin concentrations.

Solid circles indicate no inflammation; hollow circle, those with inflammation; solid diamond, hepcidin ≤ 5.5 $\mu\text{g/L}$; and hollow diamond, hepcidin > 5.5 $\mu\text{g/L}$. Markers indicate the odds ratios while error bars indicate 95% confidence intervals and are labelled respectively. Regression models were adjusted for age, sex, and study site.

6.4.4 Little evidence that *FPN* Q248H is associated with protection from malaria

It has been proposed that the *FPN* Q248H variant protects individuals from malaria (Zhang *et al.*, 2018). The Q248H mutation was not significantly associated with severe malaria overall (OR = 0.91; 0.81, 1.01; for an effect of one or two copies of Q248H relative to wild type; $P = 0.08$) or in individual cohorts (Table 6.4). Moreover, in subgroup analyses the Q248H mutation was not significantly associated with cerebral malaria (OR = 0.90; 0.77, 1.06; $P = 0.21$; estimated across cohorts), severe malarial anaemia (OR = 0.90; 0.71, 1.16; $P = 0.42$) or mortality (OR = 1.00; 0.78, 1.29; $P = 0.99$, Table 6.4). Inclusion of genotypes at the sickle-cell causing variant rs334, which is strongly associated with malaria susceptibility in these populations (Taylor, Parobek and Fairhurst, 2012), did not affect these results. The prevalence of uncomplicated malaria among community-based children also did not differ by Q248H (OR = 1.06; 95% CI 0.68, 1.64; $P = 0.79$; Table 6.4). Moreover, parasite density in children with either uncomplicated or severe malaria did not differ by the Q248H mutation (Figure 6.6).

Table 6.4. Association between *FPN* Q248H and uncomplicated or severe malaria

| Outcome | All | Q248H ¹ | WT | RR / OR (95% CI) | P value |
|---|--------------------|--------------------|-------------------|---------------------|---------|
| Malaria susceptibility ² | | | | | |
| <i>Uncomplicated malaria</i> | 260/2550 (10.2%) | 25/232 (10.8%) | 235/2318 (10.1%) | 1.06 (0.68, 1.64) | 0.79 |
| <i>Severe malaria</i> ³ | 5489/11982 (45.8%) | 658/1519 (43.3%) | 4831/10463(46.1%) | 0.91 (0.81, 1.01) | 0.08 |
| (The Gambia ⁴) | 2419/4910 (49.3%) | 225/479 (47.0%) | 2194/4431 (49.5%) | 0.88 (0.73, 1.07) | 0.21 |
| (Malawi ⁴) | 1023/2345 (43.6%) | 174/419 (41.5%) | 849/1926 (44.1%) | 0.89 (0.72, 1.10) | 0.28 |
| (Kenya ⁴) | 1446/2924 (49.5%) | 191/405 (47.2%) | 1255/2519 (49.8%) | 0.98 (0.79, 1.22) | 0.87 |
| (Ghana ⁵) | 601/1803 (33.3%) | 68/216 (31.5%) | 533/1587 (33.6%) | 0.83 (0.59, 1.17) | 0.29 |
| <i>Cerebral malaria</i> ³ | 1948/7971 (24.4%) | 249/1043 (23.9%) | 1699/6928 (24.5%) | 0.90 (0.77, 1.06) | 0.21 |
| (The Gambia ⁴) | 758/3676 (20.6%) | 60/356 (16.9%) | 698/3320 (21.0%) | 0.73 (0.55, 0.99) | 0.04 |
| (Malawi ⁴) | 644/2057 (31.3%) | 116/373 (31.1%) | 528/1684 (31.4%) | 0.95 (0.75, 1.22) | 0.71 |
| (Kenya ⁴) | 546/2238 (24.4%) | 73/314 (23.2%) | 473/1924 (24.6%) | 1.00 (0.75, 1.34) | 0.99 |
| <i>Severe malarial anaemia</i> ³ | 735/7971 (9.2%) | 82/1043 (7.9%) | 653/6928 (9.4%) | 0.90 (0.71, 1.16) | 0.42 |
| (The Gambia ⁴) | 428/3676 (11.6%) | 43/356 (12.1%) | 385/3320 (11.6%) | 0.96 (0.68, 1.35) | 0.82 |
| (Malawi ⁴) | 91/2057 (4.4%) | 12/373 (3.2%) | 79/1684 (4.7%) | 0.69 (0.37, 1.29) | 0.24 |
| (Kenya ⁴) | 216/2238 (9.7%) | 27/314 (8.6%) | 189/1924 (9.8%) | 0.93 (0.60, 1.43) | 0.74 |
| <i>Malaria-related death</i> ³ | 677/4669 (14.5%) | 86/571 (15.1%) | 591/4098 (14.4%) | 1.00 (0.78, 1.29) | 0.99 |
| (The Gambia ⁴) | 309/2335 (13.2%) | 27/219 (12.3%) | 282/2116 (13.3%) | 0.91 (0.59, 1.39) | 0.65 |
| (Malawi ⁴) | 200/1018 (19.6%) | 37/173 (21.4%) | 163/845 (19.3%) | 1.14 (0.76, 1.71) | 0.51 |
| (Kenya ⁴) | 168/1316 (12.8%) | 22/179 (12.3%) | 146/1137 (12.8%) | 0.94 (0.58, 1.52) | 0.80 |

¹Heterozygotes and homozygotes²Uncomplicated malaria was defined as *P. falciparum* positive slide measured in community-based cohorts in Uganda, The Gambia, Burkina Faso, and Kenya. Severe malaria was defined as positive for *P. falciparum* parasites plus clinical features of severe malaria (8), including diagnosis of cerebral malaria, severe malarial anaemia, and other clinical symptoms.³Relative risk, confidence interval, and P value are computed by fixed-effect meta-analysis of estimates from the three case-control cohorts and (where applicable) the Ghanaian trios.⁴Association analysis estimated by logistic regression adjusted for the first five principal components. For severe malaria and malaria-related death, results reflect binomial logistic regression of the phenotype compared to controls. For severe malaria sub-phenotypes, results reflect multinomial logistic regression of cerebral malaria, severe malarial anaemia, and other severe malaria cases compared to controls. A dominant mode of effect is assumed.⁵Counts reflect numbers of probands (affected children) and parents in 608 Ghanaian trios. Relative risk, confidence interval and P-value are computed using a transmission disequilibrium test. A dominant mode of effect is assumed.

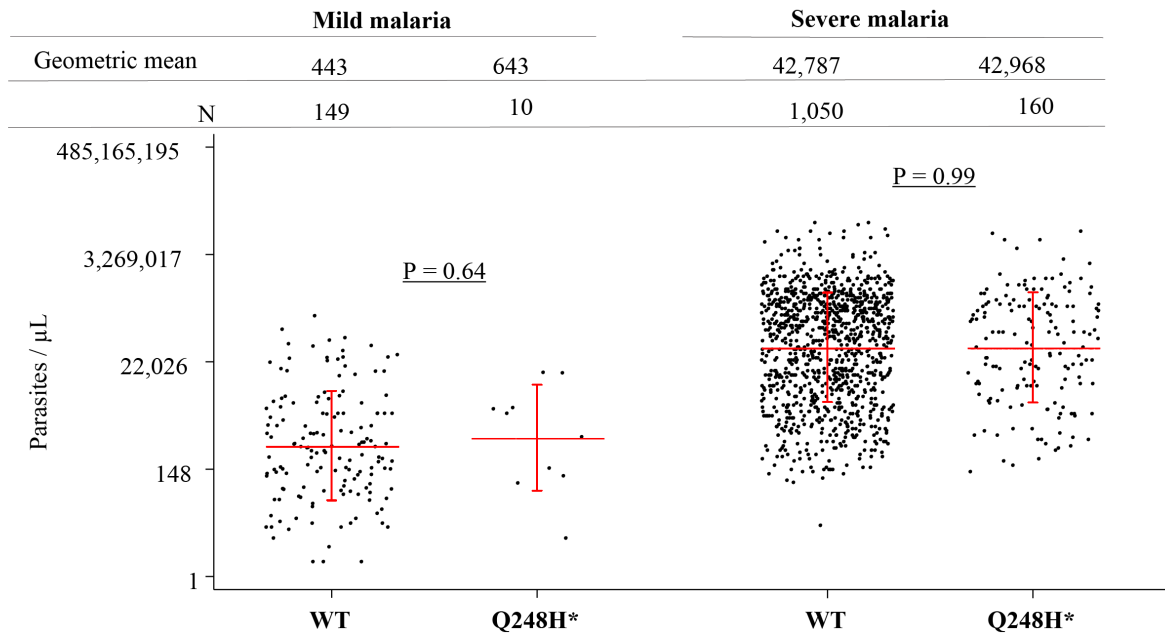


Figure 6.6. Effect of *FPN* Q248H variant on *P. falciparum* parasite density.

P. falciparum parasite densities in all community-based children with uncomplicated malaria and in hospitalised Kenyan children with severe malaria stratified by carriage of Q248H. Parasite densities are geometric means from log-transformed data. N = malaria cases. P values were derived from regression models adjusted for age and sex. *Q248H heterozygotes and homozygotes combined.

6.5 Discussion

The *FPN* Q248H variant is prevalent in African populations, and is thought to have been naturally selected to confer protection against malaria by increasing iron export from red blood cells (Zhang *et al.*, 2018). In this Chapter, I evaluated the effect of the Q248H mutation on anaemia, iron status, and malaria infection. I found that the mutation was associated with modest protection against anaemia and iron deficiency but little evidence that it protects against malaria infection.

The Q248H mutation was associated with a modest reduction in the risk of anaemia and modestly improved haemoglobin concentrations. The Q248H variant has previously been

associated with trends towards both lower and higher haemoglobin concentrations in studies of small sample size (Figure 6.3 and Table 6.3). A meta-analysis of the current and previous studies indicates an overall increase in haemoglobin concentrations in Q248H carriers (Figure 6.3). Higher haemoglobin concentrations might be due to higher iron efflux from Q248H erythrocytes protecting the cell from haemolysis (Zhang *et al.*, 2018). Indeed, I observed higher concentrations of haptoglobin among Q248H carriers, suggesting reduced haemolysis. These findings are consistent with a previous report in a mouse model, which demonstrated that ferroportin, by reducing intracellular accumulation of iron and oxidant species, prevents haemolytic anaemia and maintains integrity of red blood cells (Zhang *et al.*, 2018).

I further hypothesised that reduced hepcidin-induced degradation of ferroportin among Q248H carriers might lead to improved iron absorption by duodenal enterocytes and iron recycling by macrophages. Consistent with this hypothesis, I observed a trend towards improved iron status among children carrying the mutation corroborating findings from other studies (Gordeuk *et al.*, 2003; Kasvosve *et al.*, 2005, 2015; Rivers *et al.*, 2007). ZPP concentrations were also significantly lower among Q248H individuals suggesting no evidence for iron deficient erythropoiesis, as previously suggested (Zhang *et al.*, 2018). These findings suggest that the Q248H mutation is associated with lower risk of iron deficiency.

It has been proposed that *FPN* Q248H protects from malaria by depriving the parasite of iron within the red blood cell (Zhang *et al.*, 2018). However, I did not find a significant association between the mutation and malaria susceptibility. These data indicate that any protection afforded by the Q248H mutation in African populations is weak, with previous estimates of effect size (Zhang *et al.*, 2018) not generalising to the phenotypes and populations considered here. Additionally, the current study included 658 Q248H children with severe malaria, in

contrast to a previous report with a limited sample size of 13 Q248H children with uncomplicated malaria and 11 Q248H women with placental malaria (Zhang *et al.*, 2018). A possible explanation for a lack of protection may be due to the opposing effects of Q248H mutation on erythrocyte iron levels versus serum iron levels. This is because by increasing erythrocytic iron export the mutation may deny the parasite of iron (Zhang *et al.*, 2018). Conversely, by increasing iron export by duodenal enterocytes (Nekhai *et al.*, 2013), the mutation may increase serum iron levels and increase parasite growth.

Finally, I found that the frequency of Q248H was highest in South African children, a population which has historically experienced low malaria transmission. Moreover, further investigation of the relationship between historical rates of *P. falciparum* transmission (1900 - 2015) and the minor allele frequency of Q248H mutation showed a lack of correlation (Muriuki, Mentzer, Band, *et al.*, 2019). This suggests that *P. falciparum* prevalence in the population is not associated with the mutation and arguing against selective pressure due to malaria.

6.6 Conclusion

Using large-scale genomic datasets from African populations, I found that the Q248H mutation was associated with modest reductions in the risk of anaemia and iron deficiency, putatively due to reduced haemolysis and modestly improved iron status in Q248H carriers. These findings in large epidemiological studies build support for proposed biological pathways observed in mouse models (Zhang *et al.*, 2018), although the observed effect sizes are small, and may not be clinically significant at an individual level. Finally, I found little evidence that the Q248H mutation protects against malaria and possibly no evidence of positive selection of the variant attributable to malaria exposure.

CHAPTER SEVEN: CONCLUDING REMARKS AND RECOMMENDATIONS

Through this thesis, I investigated the complex relationship between iron deficiency and malaria. First, my findings show that iron deficiency is likely to be more common than can be estimated using the WHO definition since malaria and inflammation obscure the true burden of iron deficiency. I have also shown that transferrin saturation <11% may be a good indicator of the prevalence of iron deficiency in African children. Transferrin saturation best predicted the adjusted prevalence of iron deficiency and was also least influenced by malaria and inflammation. Accurate and reliable measurements of iron deficiency are necessary for the planning and monitoring of interventions to prevent and treat iron deficiency. Anaemia is used as a proxy for iron deficiency. However, I have shown that haemoglobin concentrations perform poorly in predicting a more accurate estimate of the prevalence of iron deficiency possibly because there are multiple overlapping factors that influence haemoglobin concentrations (Foote *et al.*, 2013). My findings also suggest that improved control of malaria and other infections would also improve assessment of the prevalence of iron deficiency.

Second, my findings suggest that malaria is an important cause of iron deficiency. Although iron supplements are the mainstay for treating iron deficiency (World Health Organization, 2016), there is a long-standing concern regarding their safety since iron may increase the risk of infections such as malaria (Sazawal *et al.*, 2006). Furthermore, since prolonged exposure to malaria leads to chronically raised hepcidin concentrations and blockade of iron absorption (Cercamondi *et al.*, 2010; Glinz, Hurrell, Righetti, *et al.*, 2015), oral iron supplements may not benefit iron-deficient children. Unabsorbed iron is also likely to disturb gut microbiota causing gastrointestinal disorders (Paganini and Zimmermann, 2017). Thus, my findings suggest a paradigm shift in the management of iron deficiency. That is, by improving control of malaria (and other infections) we can also address iron deficiency. Specifically, my findings suggest

that an intervention that halves the incidence of uncomplicated malaria would reduce 47% of the burden of iron deficiency.

Third, my findings suggest that iron deficiency may be protective against malaria risk in agreement with previous longitudinal studies (Nyakeriga *et al.*, 2004; Gwamaka *et al.*, 2012; Jonker *et al.*, 2012; Barffour *et al.*, 2017). In other words, children who are iron replete may be at an increased risk of malaria infection. Previous longitudinal studies largely reported the effect of a ferritin-based definition of iron deficiency on malaria risk. In this thesis, I analysed the effect of a wide range of iron biomarkers on subsequent risk of malaria infection. I found that concentrations of ferritin and transferrin saturation were positively associated with increased malaria risk but variation in hepcidin, sTfR, and haemoglobin or anaemia was not associated with altered malaria risk. This might reflect differences in the relationship between the iron biomarkers and the mechanisms of iron acquisition by malaria parasites. However, since my analyses and those of others (Nyakeriga *et al.*, 2004; Gwamaka *et al.*, 2012; Jonker *et al.*, 2012; Barffour *et al.*, 2017) are observational, the observed association does not prove causality.

I finally used *FPN* Q248H mutation that is associated with iron overload in previous studies (Gordeuk *et al.*, 2003; Kasvosve *et al.*, 2005; McNamara, Gordeuk and MacPhail, 2005; Masaisa *et al.*, 2012) to determine whether the mutation is also associated with increased malaria risk. Moreover, the *FPN* Q248H mutation predominantly occurs in African populations and is thought to be selected due to malaria exposure (Zhang *et al.*, 2018). My findings show that the Q248H mutation is associated with modestly improved iron status but that did not translate to increased malaria risk. Furthermore, the mutation occurred at highest prevalence in South African children, a cohort without historical malaria transmission thus arguing against a

possible selective pressure of the mutation in Africans to protect from malaria infection. It is possible that this mutation is preserved in Africans to protect against anaemia due to historical consumption of poor dietary iron. However, further research is required to understand the evolutionary benefits of the Q248H mutation in African populations.

Taken together, my findings echo the fact that there are complex interactions between iron deficiency and malaria. I found that malaria may be an important cause of iron deficiency. Conversely, (although inconclusive) iron deficiency may protect from malaria infection although anaemia or low haemoglobin concentrations are not protective. So far, these findings suggest a focus on malaria control to address both public health problems. However, future studies should investigate whether the casual relationship between iron deficiency and malaria is bidirectional.

7.1 Limitations and future directions

There are several limitations to the work presented in this thesis that warrant future research. Although the regression-correction method that I applied to estimate the ‘true’ burden of iron deficiency performs better than other methods (Namaste, Rohner, *et al.*, 2017), it is important to validate this approach. Future work should compare the prevalence estimates from the regression-correction approach and estimates obtained using the gold standard method of staining bone marrow aspirate for iron. Furthermore, the regression-correction approach could be validated by comparing prevalence estimates of regression modelling before and during / after infections. Although transferrin saturation <11% best predicted the regression-corrected estimate of iron deficiency, it had an area under the curve of only 0.77. There is therefore a need to identify easy to use, more sensitive and specific markers of iron status that are not influenced by infection.

In Chapter Four, I applied Mendelian randomisation to infer whether malaria causes iron deficiency. This method has strengths in that it avoids many of the biases that influence observational studies and provides causal effects, similar to randomised controlled trials. I used sickle cell trait as an instrumental variable to proxy malaria exposure and found that African children carrying sickle cell trait were protected from iron deficiency. I did not find a statistically significant effect of sickle cell trait in non-malaria-exposed African-American adults suggesting that the protective effect of sickle cell trait may be through malaria. However, there is a need to test whether sickle cell trait might influence iron deficiency among non-malaria-exposed African children. In addition, data comparing the degree of protection afforded by sickle cell trait against iron deficiency in populations with varying incidence of clinical malaria would be important. I would expect sickle cell trait to have a large protective effect in populations where the incidence of malaria is high. This proof of concept should also be extended to other causes of chronic infection, which may contribute to the burden of iron deficiency through inflammation-induced up-regulation of hepcidin. This would provide evidence for an integrated approach in which control of malaria and other infections could be utilised as an additional strategy to improve the iron status of children. Future research should also confirm my findings through conducting intermittent preventative treatment trials of malaria to evaluate the effect of malaria treatment on iron status.

Finally, although iron deficiency is consistently associated with protection from malaria risk (Nyakeriga *et al.*, 2004; Gwamaka *et al.*, 2012; Jonker *et al.*, 2012; Barffour *et al.*, 2017; Muriuki, Mentzer, Kimita, *et al.*, 2019), reverse causality is a possibility whereby the observed effect may be due to malaria-induced acute response of iron markers (Aguilar *et al.*, 2012; Righetti *et al.*, 2013). Future studies should infer causality using approaches such as Mendelian

randomisation and randomised controlled trials. In Mendelian randomisation analyses, a prerequisite step would be to conduct a genome-wide association study (GWAS) of iron status in African children to identify genetic variants that reliably influence iron status in this population. This is because the common genetic variants from European GWAS of iron status (Benyamin *et al.*, 2009, 2014; McLaren *et al.*, 2011) do not appear to replicate in African populations (Gichohi-Wainaina *et al.*, 2016). This limits the transferability of the European iron GWAS to populations of African ancestry. The next step would be to validate the identified genetic variants for use as instrumental variables in Mendelian randomisation analyses involving malaria case-controls in the same population. If genetic variants that are associated with improved iron status are also associated with malaria risk, then this would suggest that a child's iron status influences their risk of malaria infection. To assess how improving baseline iron status through iron supplementation would influence malaria infection, a randomised controlled trial would be necessary. Lastly, to disentangle the causal direction between iron deficiency and malaria, a bidirectional or network Mendelian randomisation study would be necessary (Davey Smith and Hemani, 2014).

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